Article

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Demethylation of TIMP2 and TIMP3 Inhibits Cell Proliferation, Migration, and Invasion in Pituitary Adenomas

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Objective: Tissue inhibitors of matrix metalloproteinases (*TIMPs*) are prognostic markers in cancers. However, the role of *TIMPs* in DNA methylation during invasive pituitary adenoma (PA) remains unclear. The purpose of this study was to assess the effects of TIMP2 and TIMP3 promoter demethylation on the proliferation, migration, and invasion of invasive PA cells.

Methods: Methylation-specific polymerase chain reaction (PCR), quantitative PCR, and western blots were used to analyze the promoter methylation and expression of TIMP1-3. Cell counting kit-8 (CCK-8), wound healing, and transwell assays were carried out to determine the effects of TIMP2 and TIMP3 demethylation.

Results: TIMP1-3 showed downregulated expression in invasive PA tissues and cell lines ($p < 0.05$). The low expression of TIMP1-3 was due to promoter methylation of these genes ($p < 0.05$). The results showed that downregulation of TIMP2 and TIMP3 can promote cell proliferation, migration, and invasion ($p < 0.05$), whereas overexpression of TIMP2 and TIMP3 can inhibit cell proliferation, migration, and invasion ($p < 0.05$). After treatment with 5-azacytidine (5-AzaC), the cell activity decreased, the proliferation rate decreased, and the invasion ability weakened ($p < 0.05$). Treatment with 5-AzaC increased TIMP2 and TIMP3 expression and decreased DNA (cytosine-5-)-methyltransferase 1 (*DNMT1*), DNMT3a, and DNMT3b expression ($p < 0.05$).

Conclusions: We showed that DNA methylation causes the silencing of TIMP2 and TIMP3 in invasive PA, it can also lead to malignant cell proliferation and cause pathological changes, whereas the use of 5-AzaC can inhibit the methylation process and can inhibit cell proliferation. Our results provide a novel method for clinical diagnosis and prevention of invasive PA.

Keywords: pituitary adenoma; TIMPs; DNA methylation; 5-AzaC

Introduction

Pituitary adenoma (PA) is the most commonly diagnosed benign neuroendocrine tumor globally, leading to significant morbidity and increased mortality. PA is the third most common intracranial neoplasm, accounting for 10–15%, with a total prevalence of around 20% in the general population as estimated through radiology and autopsy [1–3]. Symptoms associated with PA are caused by effects on the endocrine system and by intracranial tumor occupation, resulting in side effects such as affective disorders, sexual dysfunction, obesity, and diabetes [4,5]. Approximately 30% of PAs are invasive and can invade the adjacent sphenoid and cavernous sinuses. In cases of extensive local invasion, complete resection is required but remains challenging [5,6]. Given the urgent need for early diagnosis and treatment, unveiling the molecular mechanisms of invasive PA is critical [7].

Matrix metalloproteinases (*MMPs*) are members of the endopeptidase family, and play a crucial role in the degradation of the natural extracellular matrix and basement membrane, allowing the proliferation, migration, and invasion of tumor cells [8]. Tissue inhibitors of matrix metalloproteinases (*TIMPs*) are inhibitors that specifically inhibit the active form of MMPs [9–11] and act as tumor suppressors in several tumors. TIMP2, as a possible antagonist of MMP2, suppresses metastasis in cervical cancer cell invasion [12]. In oral squamous cell carcinoma, TIMP3 acts as a potential biomarker that can predict tumor stage and T-status [13]. TIMP3 overexpression in hepatocellular carcinoma suppresses cell proliferation, enhances apoptosis, and inhibits migration and invasion [14]. TIMPs also stimulate tumor suppressor messengers, thereby suppressing tumors in lung, prostatic, and colorectal cancer [15]. However, the precise biological functions and potential mechanisms of TIMP1-3 in the PA process are not fully understood.

Epigenetic events in cancer have been well studied [16]. For instance, DNA methylation of eight genes was associated with cervical cancer recurrence [17], and *APC* promoter methylation might be a marker for gastric cancer diagnosis [18]. Indeed, aberrant DNA methylation of tumor suppressor encoding genes has been often mentioned

in these studies [19,20]. The previous study has shown that DNA methylation of tumor suppressor (*TSHZ3*) plays a crucial role in colorectal cancer [21]; a tumor suppressor (*RAP1GAP*) can be regulated by DNA methylation in thyroid cancer [22]; and Retinoblastoma protein-interacting zinc finger protein 1 (*RIZ1*), as a tumor suppressor, can be affected in cervical cancer [23]. Several studies have demonstrated DNA methylation-induced changes in TIMPs expression to be one of the most significant steps in tumor growth, invasion, and metastasis. For example, TIMP2 expression is inhibited by methyltransferase enhancer of zeste homolog 2 (*EZH2*) in ovarian cancer, relieving MMP repression and promoting invasion and migration [24]. Cao and colleagues [25] also reported that TIMP3 promoter methylation is positively associated with the risk of gastric cancer, and TIMP3 might be a biomarker for gastric cancer. Another study by Li *et al*. [26] reported that in chronic periodontitis, methylation levels were lower for MMP-9 but higher for TIMP-1 in female patients compared to male patients, and TIMP-1 methylation levels gradually diminished with age. Therefore, these studies suggest a possibility for a close relationship between the abnormal expression of TIMPs in invasive PA and transcriptional silencing involved in DNA methylation. However, whether DNA methylation of TIMP1-3 can alter the malignant course of PA has not been reported.

In this paper, we aimed to investigate the effect of TIMP2 and TIMP3 demethylation on the malignancy of PA cells. We explored the expression patterns and DNA methylation of TIMPs in clinical samples (The Second Affiliated Hospital of Guilin Medical University) and PA cell lines. We also elucidated the cellular functions of TIMPs in PA cell proliferation, migration, and invasion by RNA interference or 5-azacytidine (5-AzaC) treatment. Therefore, the investigation of the role of TIMPs DNA methylation on the progression of invasive PA might provide a novel method for clinical diagnosis and prevention of invasive PA.

Materials and Methods

Collection of Tissue Samples

Human invasive or non-invasive PA tissue samples were obtained from 20 cases of PA patients between March 2020 and December 2020 from The Second Affiliated Hospital of Guilin Medical University. Invasive PA was defined as Knosp classification grades III–IV and Hardy-Wilson classification grades III–IV [27,28]; non-invasive PA was defined as a tumor confined to the tissue without any compression of surrounding structures [29]. In addition, normal pituitary tissues from the thin layer around the adrenocorticotropic hormone (*ACTH*) microadenocarcinoma were obtained from the 20 cases to serve as a control group. Patients were excluded based on the following criteria: patients having undergone previous gamma knife radiotherapy or surgery; having incomplete data; postoperative histopathological diagnosis of non-pituitary adenoma; combined renal disease or cardiovascular or cerebrovascular disease; or having uncooperative information after consultation. Inclusion criteria for patients were: first, invasive PA met the Knosp grade and Hardy-Wilson grade, and non-invasive PA was confined to the tissue without compressing the surrounding structures of the tumor; second, patients who had not received gamma knife radiation therapy or surgery, had complete data, had a postoperative histopathology diagnosis of pituitary adenoma, and had no history of renal disease or cardiovascular or cerebrovascular disease. All specimens were obtained from The Second Affiliated Hospital of Guilin Medical University, and ethical approval was granted by its Research Ethics Committee (2014010-2-5). Before tissue collection, informed consent was received from each PA patient involved in this study. Once obtained, these patient samples were quickly transferred into liquid nitrogen and stored in a –80 °C freezer.

Cell Culture

The rat PA cell lines MMQ (CRL-10609), GH1 (CCL-82.1), GH3 (CCL-82.1), RC-4B/C (CRL-1903), and normal pituitary cells (CRL-1395) were purchased from the ATCC (Manassas, VA, USA) and underwent morphological assessment and surface marker identification using flow cytometry. Mycoplasma testing was performed. ATCC-formulated F-12K medium (21127-022, Invitrogen, Carlsbad, CA, USA) was used for the routine culture of MMQ, GH1, and GH3, and Dulbecco's Modified Eagle's medium (C11960500BT, DMEM, Invitrogen, Carlsbad, CA, USA) was used for routine culture of RC-4B/C and normal pituitary cells. Fetal bovine serum (A5669701, Thermo Fisher Scientific, Waltham, MA, USA) was added to all cultured media at 10% bulk concentration, and penicillin and streptomycin at 100 units/mL. These cells were cultured in a 37 °C incubator (Cytomat^{TM} 10 C450, Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO_2.

Cell Treatment and Transfection

Before treatment, 1 × 10^6 GH3 cells were inoculated in a 6-well plate. Then 10 µmol/L 5-AzaC (PHR1911, Sigma-Aldrich, St. Louis, MO, USA) were added into the cultured medium for 1 day at 37 °C. Small interfering RNA (siRNA) of *TIMP2* (siTIMP2, 5′GCAATGCAGACGTAGTGATCA 3′), *TIMP3* (siTIMP3, 5′ GCAGACAGACGCCAGG TATTT 3′), and control siRNA (siNC) were designed and synthesized by GenePharma (Shanghai, China) and transfected into GH3 cells at a concentration of 10 nM. The eukaryotic expression vector of *TIMP2* or *TIMP3* was constructed by RiboBio (Guangzhou, China) by subcloning the coding sequence region of *Homo sapiens* TIMP2 or TIMP3 into pcDNA3.0 vector (Invitrogen, Carlsbad, CA, USA). The plasmid vectors pcDNA3.0-TIMP2, pcDNA3.0-TIMP3, or pcDNA3.0 were transfected into the

Table 1. Primers used for quantitative real-time PCR analysis.

Gene	Primer sequence 5′-3′	PS (bp)
Human-*TIMP1*	F: AGAGTGTCTGCGGATACTTCC R: CCAACAGTGTAGGTCTTGGTG	169
Human-*TIMP2*	F: AAGCGGTCAGTGAGAAGGAAG R: GGGGCCGTGTAGATAAACTCTAT	136
Human-*TIMP3*	F: CAGGTCGCGTCTATGATGGC R: AGGTGATACCGATAGTTCAGCC	109
Human-*GAPDH*	F: TGTTCGTCATGGGTGTGAAC R: ATGGCATGGACTGTGGTCAT	154
Rat-*TIMP2*	F: TTGGAGGAAAGAAGGAATA R: GTAGCATGGGATCATAGGG	185
Rat-*TIMP3*	F: CCTTTGGCACTCTGGTCTA R: TCAGCAGGTACTGGTATTT	135
Rat-*GAPDH*	F: CGACCCCTTCATTGACCTC R: GCCAGTAGACTCCACGACATAC	151

PCR, polymerase chain reaction; *TIMP*, tissue inhibitors of matrix metalloproteinases; F, forward; R, reverse; PS, product size; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

GH3 cells. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used as the cell transfection reagent following the manufacturer's instructions. Cells were harvested and subjected to the subsequent experimental procedure two days after transfection.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA from tissue specimens or cell lines was extracted by TRIzol reagent (15596018, Invitrogen, Carlsbad, CA, USA). M-MLV reverse transcriptase (170-8890, Bio-Rad, Minneapolis, MN, USA) was used for reverse transcription via oligo primer extension (3806, TaKaRa, Dalian, China). Quantitative PCR was performed using a qPCR Mix kit (DBI-2243, DBI Bioscience, Leipzig, Germany) on the Mx3000P Real-time PCR Amplifier (Agilent, Santa Clara, CA, USA). The primer sequence is listed in Table 1. The $2^{-\Delta\Delta Ct}$ method was utilized for mRNA expression calculation, which was then normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA.

Western Blot Analysis

RIPA lysis buffer (R0278, Roche, Basel, Switzerland) was used for total protein extraction from tissue samples, and the bicinchoninic acid (BCA) assays (BCA1, Sigma, St. Louis, MO, USA) were performed for protein quantitation. The loading amount of each protein sample subjected to SDS-PAGE was approximately 30 µg. After gel electrophoresis, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (1620177, Bio-Rad, Minneapolis, MN, USA) using the wet electrophoresis transfer method. Membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBST) buffer (T9039, Sigma, St. Louis, MO, USA) for 1 hour at room temperature. Membranes were then subjected to overnight incubation with corresponding primary antibodies procured from Abcam (Cambridge, UK) against TIMP1 (1:1000, ab61224), TIMP2 (1:2000, ab180630), TIMP3 (1:4000, ab39184), DNA (cytosine-5-)-methyltransferase 1 (DNMT1) (1:1000, ab188453), DNMT3a (1:2000, ab227823), DNMT3b (1:1500, ab2851), and GAPDH (1:10,000, ab181602) (Abcam, Cambridge, UK) at 4 °C. TBST was used as a wash buffer to wash the membranes 3 times every 5 min; membranes were then subjected to incubation with a horseradish peroxidase-linked secondary antibody (1:20,000, BA1054, Boster, Wuhan, China) for 1 hour at room temperature. After incubation, protein signals on the membranes were visualized using a chemiluminescence kit (sc-2048, Santa Cruz, Dallas, TX, USA). The immunostained protein bands were detected using a chemiluminescence system (BeyoImager™ 600, Beyotime Biotechnology, Shanghai, China) and ImageJ software (v1.8.0, U. S. National Institutes of Health, Bethesda, MD, USA) was used to measure the gray values of the bands for statistical analysis. Statistical analysis was carried out among the groups by each protein band and the gray value ratio of internal parameter GAPDH.

Methylation-Specific PCR (MSP)

Genomic DNA was extracted using the High Pure PCR Template Preparation kit (No.117968282001, Roche, Basel, Switzerland). Online software (website: http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) was utilized to predict the sequence of promoter 5′-C-phosphate-G-3′ (CpG) islands of the relevant genes. The

EpiTect Bisulfite kit (59104 ea, Qiagen, Hilden, Germany) was used to treat DNA samples containing sodium bisulfite and the EZ DNA Methylation-Gold kit (D5005, Zymo Research, Orange, CA, USA) was used for DNA modification. Design of the methylated or unmethylated specific primer sequences to amplify bisulfite DNA was conducted using Methyl Primer Express v1.0 software (No.4376041, Thermo Fisher Scientific, Waltham, MA, USA) (Table 2). MSP products of the methylated genes were analyzed using electrophoresis with 2% agarose gels. Episcope® CpG methylase-treated HCT116 gDNA (Clontech, Mountain View, CA, USA) was used as the positive control, and distilled water was used as the negative control [30].

Table 2. Methylated or un-methylated specific primer sequences are used for MSP.

Gene	Primer sequence 5′-3′	PS (bp)
TIMP2	M-F: ATTATAGGTATTAGATGGGTTGCGA M-R: TCGAAAAACTCCTACTTAAAAAACG	214
TIMP2	U-F: ATTATAGGTATTAGATGGGTTGTGA U-R: CAAAAAACTCCTACTTAAAAAACACC	213
TIMP3	M-F: GATGTATACGGGGTTGTGTAATTTC M-R: ATATCGATCCAAAAACACTCGTT	168
TIMP3	U-F: TGTATATGGGGTTGTGTAATTTTGT U-R: ACATATCAATCCAAAAACACTCATT	168

M-F, methylated forward; M-R, methylated reverse; U-F, un-methylated forward; U-R, un-methylated reverse; PS, product size; MSP, Methylation-specific PCR.

Cell Counting Kit-8 (CCK-8) Assay

The treated or transfected GH3 cells were first seeded in 96-well plates at about 3000 cells per well. Cells were cultured overnight at 37 °C, and then CCK-8 (CK-04-500T, Dojindo Laboratories, Kumamoto, Japan) reagent was added to the cultured medium to determine cell proliferation capacity. Ten µL of CCK-8 solution was added into each well's cultured medium at the indicated time after seeding and treated for 2 hours at 37 °C. A microplate reader (Bio-Rad 680, Bio-Rad, Minneapolis, MN, USA) was used to detect the optical density value at 450 nm.

Wound Healing Assay

GH3 cells were seeded in 6-well plates at about 4000 cells per well and cultured to about 90% confluence. A straight scratch through the monolayer was created artificially by 100 µL sterile pipette tips. After wounding in 5 randomly chosen fields, images displaying the same location were observed at 0 and 48 hours using a stereomicroscope (Olympus, Tokyo, Japan). Relative average migration distance was calculated as follows: (Width 0 h – Width 48 hours)/Width 0 h × 100%.

Cell Invasion Assay

50 µL of diluted Matrigel (1:8; E6909, Millipore, St. Louis, MO, USA) were first pre-paved on the transwell chambers (PTSP06H48; Millipore, St. Louis, MO, USA) for 30 min at 37 °C. Then the processed GH3 cells (200 µL, 3 × 10^5 cells/well) were supplemented into the upper transwell chambers, and 500 µL of fresh medium containing 10% FBS was added into the lower chamber. After incubation at 37 °C for 48 hours, the cells that moved from the upper chamber to the lower one were fixed and stained with 0.5% crystal violet. Images were obtained using a microscope (Olympus BX-53, Olympus, Tokyo, Japan) in 5 randomly chosen fields.

Statistical Analysis

Data were analyzed by Statistical Package for the Social Sciences (SPSS) 21.0 software (IBM Corp., Armonk, NY, USA). The data were presented as the mean ± standard deviation (SD) of at least 3 repeated experiments. A two-tailed Student's *t*-test was used to compare the continuous data for two groups, and a one-way analysis of variance (ANOVA) followed by Tukey's test was used for data with multiple groups. p values < 0.05 were considered statistically significant.

Results

TIMP1-3 Expression and Methylation Status in PA Tissues

To examine the role of TIMPs in PA, we first measured the mRNA levels of *TIMP1-3* in human PA samples by qRT-PCR. Twenty cases of invasive pituitary tumors (I), twenty cases of non-invasive pituitary tumors (N), and twenty matched controls (C) were evaluated. Compared with the controls, *TIMP1-3* expression was downregulated in both invasive and non-invasive pituitary tumors (Fig. 1A) ($p < 0.05$). Notably, compared with non-invasive pituitary tumors, the expression of *TIMP1-3* was further reduced in invasive pituitary tumors ($p < 0.001$). Results of the western blot analysis indicated that TIMP1-3 protein levels showed the same trend across tissue samples as seen in the qRT-PCR results (Fig. 1B) ($p < 0.05$).

We predicted that the methylation status of the CpG site of the TIMP1-3 promoter would lead to abnormal expression of TIMP1-3 in PA tissues. However, methylation states showed that only TIMP2 and TIMP3 contained typical CpG islands, suggesting that promoter CpG methylation may only play a role in TIMP2 and TIMP3 silencing. Next, we explored the methylation status of TIMP2 and TIMP3 to quantify the degree of methylation. The MSP assay confirmed 100% methylation of TIMP2 (Fig. 1C) and TIMP3 (Fig. 1D) in three typical cases of invasive pituitary tumors, whereas 0% methylation was observed in non-

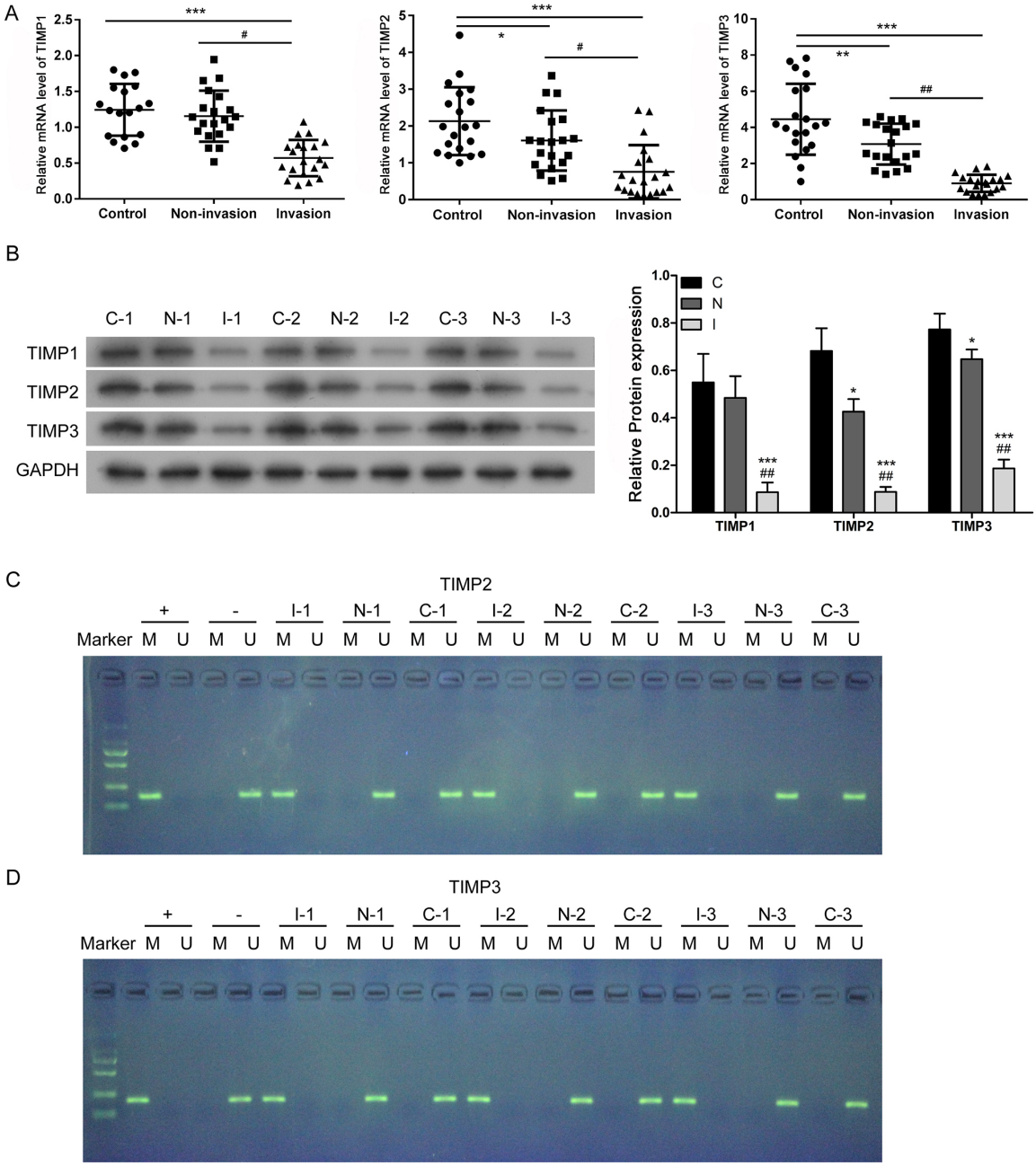

Fig. 1. TIMP2 and TIMP3 expression and methylation in pituitary adenoma (PA) tissues. (A) Quantitative real-time PCR results show the mRNA levels of *TIMP1-3* in 20 cases of invasive pituitary tumors, 20 cases of non-invasive pituitary tumors, and 20 matched control tissue specimens, n = 20. Significance was defined as *- $p < 0.05$, **- $p < 0.01$, ***- $p < 0.001$, compared with C; #- $p < 0.05$, ##- $p < 0.01$, compared with N samples. (B) Western blot analysis illustrating TIMP1-3 protein levels, evaluated in a representative set of 3 pairs of tissue specimens, n = 3. *- $p < 0.05$, ***- $p < 0.001$, compared with C; ##- $p < 0.01$, compared with N. GAPDH acted as the internal control. (C,D) MSP assays illustrate the methylation status of TIMP2 (C) and TIMP3 (D) in the three representative pairs of tissue specimens, n = 3. +, positive control; -, negative control; C, control; N, non-invasive; I, invasive; M, methylated primer; U, unmethylated primer.

invasive pituitary tumors and controls. These findings suggested that promoter methylation might cause a decrease in both TIMP2 and TIMP3 expression in invasive pituitary tumors.

TIMP2 and TIMP3 Suppress Proliferation, Migration, and Invasion in PA Cells

Quantitative PCR and western blots were carried out to monitor the mRNA and protein levels in several PA cell lines, and the biological functions of TIMP2 and TIMP3

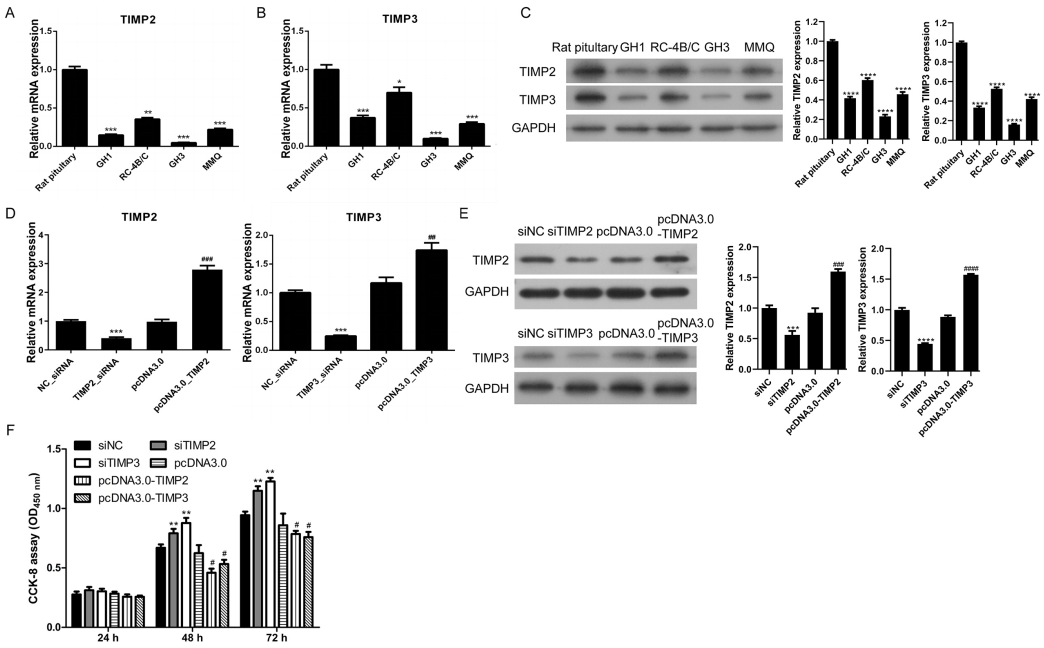

Fig. 2. TIMP2 and TIMP3 inhibit cell proliferation in pituitary adenoma cells. (A,B) Quantitative PCR and (C) Western blots were used to detect the mRNA and protein levels of TIMP2 and TIMP3 in PA cell lines, including MMQ, GH1, GH3, and RC-4B/C, along with normal rat pituitary cells, n = 3. Data are expressed as mean ± SD. *- $p < 0.05$, **- $p < 0.01$, ***- $p < 0.001$, ****- $p < 0.0001$, compared with the rat pituitary cells. GH3 cells were transfected with control small interfering RNA (siNC), siTIMP2, siTIMP3, pcDNA3.0 empty vector, pcDNA3.0-TIMP2, or pcDNA3.0-TIMP3, respectively. (D) Quantitative PCR results showing mRNA levels of *TIMP2* and *TIMP3*, n = 3. (E) Western blots show the protein levels of TIMP2 and TIMP3 in GH3 cells, n = 3. (F) Cell counting kit-8 (CCK-8) assay illustrates the cell proliferation capacity, n = 3. Data are presented as mean ± SD. **- $p < 0.01$, ***- $p < 0.001$, ****- $p < 0.0001$, compared to siNC; #- $p < 0.05$, ##- $p < 0.01$, ###- $p < 0.001$, ####- $p < 0.0001$, compared with empty vector pcDNA3.0.

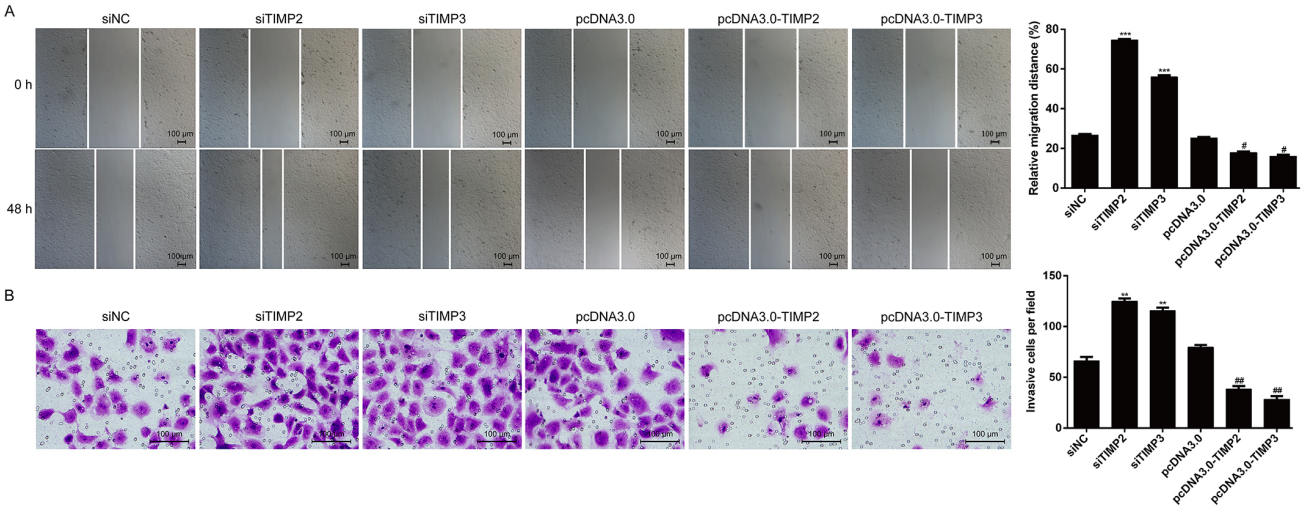

Fig. 3. TIMP2 and TIMP3 inhibit cell migration and invasion in pituitary adenoma cells. GH3 cells were transfected with siNC, siTIMP2, siTIMP3, pcDNA3.0 empty vector, pcDNA3.0-TIMP2, or pcDNA3.0-TIMP3, respectively. (A) Representative images (40× magnification) of the wound-healing assay (left panel) at indicated time points. The relative area of cell migration at 48 hours compared with that at 0 h calculated by ImageJ software in GH3 cells (right panel), n = 3. (B) Image shows GH3 cells in the transwell invasion assay that invaded the lower chamber (left panel, 200× magnification), n = 3. The right panel shows the number of cells invaded analyzed by ImageJ software. Data were presented as mean ± SD. **- $p < 0.01$, ***- $p < 0.001$, compared with siNC; #- $p < 0.05$, ##- $p < 0.01$, compared with empty vector pcDNA3.0.

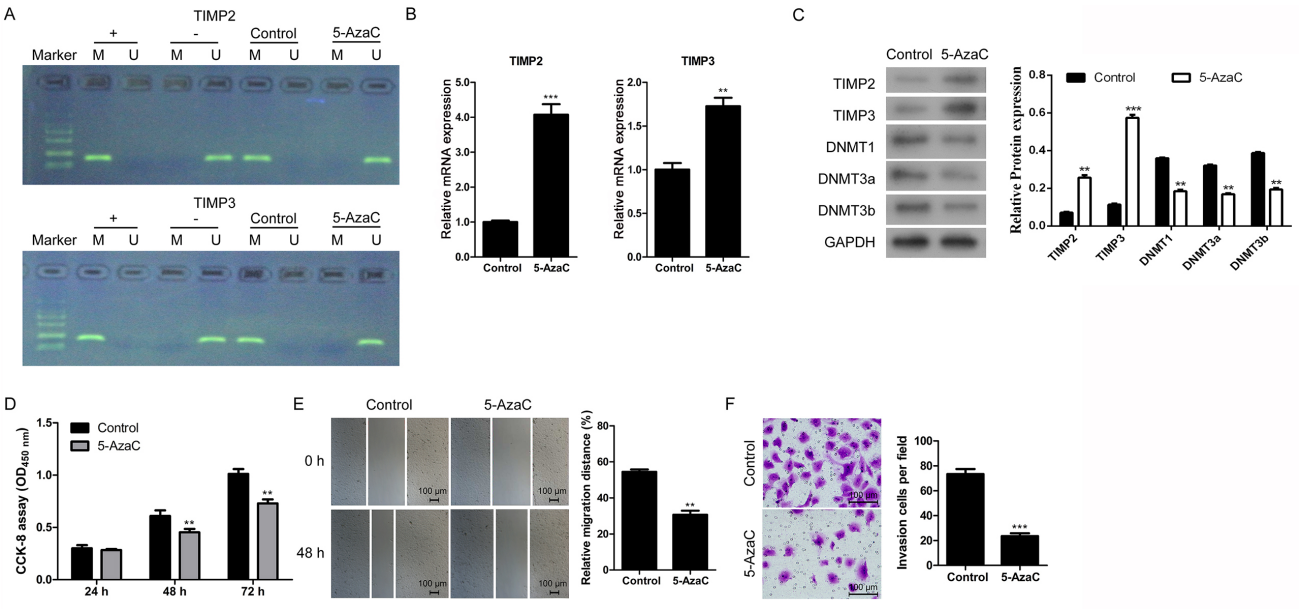

Fig. 4. Ectopic expression of TIMP2 and TIMP3 by demethylation inhibits proliferation, migration, and invasion. (A) MSP assays detecting TIMP2 and TIMP3 methylation in GH3 cells post 5-azacytidine (5-AzaC) treatment; +, positive control; -, negative control; Abbreviations: M, methylated primer; U, un-methylated primer. (B) *TIMP2* and *TIMP3* mRNA levels were detected by quantitative PCR, n = 3. (C) Western blot results show protein levels of TIMP2, TIMP3, DNMT1, DNMT3a, and DNMT3b in GH3 cells after 5-AzaC treatment, n = 3. (D) CCK-8 assays show cell viability in GH3 cells upon 5-AzaC treatment, n = 3. (E) Wound-healing and (F) Transwell invasion assays were carried out to assess migration and invasion capacity, n = 3. Data were expressed as mean ± SD. **- $p < 0.01$, ***- $p < 0.001$, compared with control. DNMT1, DNA (cytosine-5-)-methyltransferase 1.

were investigated. Compared with normal rat pituitary cells, TIMP2 and TIMP3 mRNA and protein expression levels were significantly downregulated in MMQ, GH1, GH3, and RC-4B/C PA cell lines (Fig. 2A–C) ($p < 0.05$). TIMP2 and TIMP3 showed the lowest expression in GH3 cells among all the examined PA cell lines. Thus, subsequent functional experiments were performed with the GH3 rat PA cell line.

To understand the role of TIMPS, we downregulated and overexpressed TIMP2 and TIMP3 by transfecting GH3 cells with siTIMP2, siTIMP3, or pcDNA3.0-TIMP2, pcDNA3.0-TIMP3. Quantitative PCR showed a significant decrease in the mRNA levels of *TIMP2* and *TIMP3* after siRNA interference and a significant increase after eukaryotic expression vector pcDNA3.0 overexpression in GH3 cells (Fig. 2D) ($p < 0.05$). Western blot analysis further confirmed these results (Fig. 2E) ($p < 0.05$). Knockdown of TIMP2 or TIMP3 promoted proliferation, migration, and invasion as demonstrated by CCK-8 (Fig. 2F), wound healing (Fig. 3A), and transwell invasion assays (Fig. 3B) ($p < 0.05$). Conversely, an opposite effect was observed when TIMP2 or TIMP3 was overexpressed in GH3 cells ($p < 0.05$).

Demethylation of TIMP2 or TIMP3 Inhibits Proliferation, Migration, and Invasion in PA Cells

Since both TIMP2 and TIMP3 harbor methylation potential in the promoter region, we investigated whether the expression level of TIMP2 and TIMP3 could be affected by promoter methylation. GH3 cells were treated with DNA methyltransferase inhibitor 5-AzaC *in vitro*. MSP assay results showed that the promoter regions of TIMP2 and TIMP3 in GH3 cells did not have GpG sites under 5-AzaC demethylation (Fig. 4A). The expression levels of mRNA (Fig. 4B) and protein of TIMP2 and TIMP3 were upregulated upon 5-AzaC treatment (Fig. 4C) ($p < 0.01$). The 5-AzaC treatment also suppressed the expression of DNA-methyltransferases, such as DNMT1, DNMT3a, and DNMT3b (Fig. 4C), as demonstrated by western blot analysis ($p < 0.01$). We further examined whether demethylation of TIMP2 or TIMP3 under 5-AzaC treatment resulted in similar results to TIMP2 or TIMP3 overexpression. A series of *in vitro* functional assays revealed that cell proliferation, migration, and invasion were inhibited under 5-AzaC treatment, as demonstrated by CCK-8 assays (Fig. 4D) ($p < 0.01$), wound healing assays (Fig. 4E) ($p < 0.01$), and transwell invasion assays (Fig. 4F) ($p < 0.001$). Taken together, the results suggested that the ectopic expression of TIMP2 and TIMP3 caused by promoter demethylation inhibits the proliferation, migration, and invasion of PA cells.

Discussion

In ovarian cancer cells, the reduction of TIMP2 promoter methylation causes the reactivation of TIMP2 transcription [24]. TIMP3 also possesses hypermethylation status in oropharyngeal squamous cell carcinoma cases [31]. Maleva Kostovska and colleagues [32] recently reported that TIMP3 promoter methylation might be a biomarker of BRCA1ness tumors at the epigenetic level. TIMP1-3 has been identified as a prognostic biological marker in invasive PA [33–35]. These studies mainly focused on the action and methylation status of TIMP2 and TIMP3 because only TIMP2 and TIMP3 contain typical CpG islands in PA tissues. Consistent with these findings, our results demonstrated that the expression of TIMP1-3 in PA patients was significantly downregulated in invasive PA tissues. Moreover, the promoter DNA methylation status in invasive PA tissues caused downregulation of TIMP2 or TIMP3.

Downregulation of TIMP2 or TIMP3 promoted cell proliferation, migration, and invasion, whereas overexpression of TIMP2 or TIMP3 did the opposite. TIMPs are specific protease inhibitors that negatively regulate MMP activity and are downregulated in many solid tumors, acting as tumor metastasis suppressors [36,37]. For instance, TIMP3 negatively regulates multiple aspects of the metastatic cascade in melanoma tumors [38]. Furthermore, TIMPs maintain an extracellular matrix in the human pituitary [39].

It has been reported that TIMP-1 and TIMP-2 expression levels are reduced in invasive PA [40]. Therefore, we further speculated that TIMPs might play an antitumor role in invasive PA cells. As further validation that reduced expression levels of TIMP2 or TIMP3 were closely associated with DNA methylation, we treated GH3 cells with 5-AzaC to analyze their proliferation, migration, and invasion. 5-AzaC treatment significantly suppressed the proliferation, migration, and invasion of GH3 cells. Similar results were obtained when TIMP2 or TIMP3 were overexpressed. DNMT1, DNMT3a, and DNMT3b were reduced in GH3 cells after 5-AzaC treatment, along with increased expression of TIMP2 and TIMP3. It has been previously reported that inhibition of promoter hypermethylation in lung cancer can significantly increase the expression of TIMP3 by decreasing the expression of DNMT1 [41]. Compounds other than 5-AzaC, such as natural secolignan peperomin E, are also considered effective inhibitors of DNA methylation, downregulating DNMT1, DNMT3a, and DNMT3b, and inducing promoter hypomethylation of the genes that suppress metastasis such as E-cadherin and TIMP3 in gastric cancer cells [42]. The study by Ma *et al.* [43] has shown that there is a possible function of DNMT1 and DNMT3a in promoter methylation of the genes encoding tumor suppressors, suggesting that DNMT inhibitors have the potential to develop into novel targeted therapies for invasive PA. Our results also indicated that downregulation of TIMP2 or TIMP3, due to DNA methylation and suppression of promoter hypermethylation, could be an effective way to enhance the antitumor effects of TIMPs in PA cells.

Conclusions

We have demonstrated for the first time that the decreased expression level of TIMP2 or TIMP3 in invasive PA is partly because of DNA methylation at the promoter region. RNA interference to decrease the level of *TIMP2* or *TIMP3* accelerated proliferation, migration, and invasion of GH3 cells, then was promoted in PA. Therefore, our results elucidated the underlying mechanisms and role of TIMPs in PA.

Availability of Data and Materials

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

YDY, FJH, XFW, and CQH conducted experiments. FJH and XFW, YL analyzed the data and arranged figures. YDY, FJH, XFW, CQH and YL were involved in drafting and critical revision of the manuscript. All of the listed authors approved the submitted manuscript and all authors agreed to be accountable for all aspects of this work.

Ethics Approval and Consent to Participate

Ethical approval was obtained from the Research Ethics Committee of The Second Affiliated Hospital of Guilin Medical University (2014010-2-5). Written informed consent was obtained from each patient participating in this study before enrollment and was conducted in accordance with Good Clinical Practice guidelines and the principles of the Declaration of Helsinki.

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Osman M, Wild A. Spindle Cell Oncocytoma of the Anterior Pituitary Presenting with an Acute Clinical Course Due To Intraventricular Hemorrhage. A Case Report and Review of Literature. The American Journal of Case Reports. 2017; 18: 894–901.

[2] Peng H, Liu H, Zhao S, Wu J, Fan J, Liao J. Silencing of RASSF3 by DNA hypermethylation is associated with tumorigenesis in somatotroph adenomas. PloS One. 2013; 8: e59024.

[3] Wang RQ, Lan YL, Lou JC, Lyu YZ, Hao YC, Su QF, *et al*. Expression and methylation status of LAMA2 are associated with the invasiveness of nonfunctioning PitNET. Therapeutic Advances in Endocrinology and Metabolism. 2019; 10: 2042018818821296.

[4] Asa SL, Ezzat S. The pathogenesis of pituitary tumours. Nature Reviews. Cancer. 2002; 2: 836–849.

[5] Yang Q, Li X. Molecular Network Basis of Invasive Pituitary Adenoma: A Review. Frontiers in Endocrinology. 2019; 10: 7.

[6] Scheithauer BW, Kovacs KT, Laws ER, Jr, Randall RV. Pathology of invasive pituitary tumors with special reference to functional classification. Journal of Neurosurgery. 1986; 65: 733–744.

[7] Priola SM, Esposito F, Cannavò S, Conti A, Abbritti RV, Barresi V, *et al*. Aggressive Pituitary Adenomas: The Dark Side of the Moon. World Neurosurgery. 2017; 97: 140–155.

[8] Zhou Y, Wang R, Chen SX, Wu LS, Zhu JQ. Matrix metalloproteinase 9 gene promoter region-1562 C/T single nucleotide polymorphism increases the susceptibility to hypertensive disorders of pregnancy: a meta-analysis. Clinical and Experimental Obstetrics & Gynecology. 2021; 48: 245–252.

[9] Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circulation Research. 2003; 92: 827–839.

[10] Rodríguez D, Morrison CJ, Overall CM. Matrix metalloproteinases: What do they not do? New substrates and biological roles identified by murine models and proteomics. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2010; 1803: 39–54.

[11] Gomis-Rüth FX, Maskos K, Betz M, Bergner A, Huber R, Suzuki K, *et al*. Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. Nature. 1997; 389: 77–81.

[12] Cheng Y, Geng L, Zhao L, Zuo P, Wang J. Human papillomavirus E6-regulated microRNA-20b promotes invasion in cervical cancer by targeting tissue inhibitor of metalloproteinase 2. Molecular Medicine Reports. 2017; 16: 5464–5470.

[13] Su CW, Su BF, Chiang WL, Yang SF, Chen MK, Lin CW. Plasma levels of the tissue inhibitor matrix metalloproteinase-3 as a potential biomarker in oral cancer progression. International Journal of Medical Sciences. 2017; 14: 37–44.

[14] Shen B, Jiang Y, Chen YR, Zheng HC, Zeng W, Li YY, *et al*. Expression and inhibitory role of TIMP-3 in hepatocellular carcinoma. Oncology Reports. 2016; 36: 494–502.

[15] Lin H, Zhang Y, Wang H, Xu D, Meng X, Shao Y, *et al*. Tissue inhibitor of metalloproteinases-3 transfer suppresses malignant behaviors of colorectal cancer cells. Cancer Gene Therapy. 2012; 19: 845–851.

[16] Köhler F, Rodríguez-Paredes M. DNA Methylation in Epidermal Differentiation, Aging, and Cancer. The Journal of Investigative Dermatology. 2020; 140: 38–47.

[17] Ma JH, Huang Y, Liu LY, Feng Z. An 8-gene DNA methylation signature predicts the recurrence risk of cervical cancer. The Journal of International Medical Research. 2021; 49: 3000605211018443.

[18] Zhou X, Jiao D, Dou M, Zhang W, Hua H, Chen J, *et al*. Association of APC gene promoter methylation and the risk of gastric cancer: A meta-analysis and bioinformatics study. Medicine. 2020; 99: e19828.

[19] Choi SJ, Jung SW, Huh S, Chung YS, Cho H, Kang H. Alteration of DNA Methylation in Gastric Cancer with Chemotherapy. Journal of Microbiology and Biotechnology. 2017; 27: 1367–1378.

[20] Usui G, Matsusaka K, Mano Y, Urabe M, Funata S, Fukayama M, *et al*. DNA Methylation and Genetic Aberrations in Gastric Cancer. Digestion. 2021; 102: 25–32.

[21] Zhou Y, Wang S, Yin X, Gao G, Wang Q, Zhi Q, *et al*. TSHZ3 functions as a tumor suppressor by DNA methylation in colorectal cancer. Clinics and Research in Hepatology and Gastroenterology. 2021; 45: 101725.

[22] Faam B, Ghaffari MA, Khorsandi L, Ghadiri AA, Totonchi M, Amouzegar A, *et al*. RAP1GAP Functions as a Tumor Suppressor Gene and Is Regulated by DNA Methylation in Differentiated Thyroid Cancer. Cytogenetic and Genome Research. 2021; 161: 227–235.

[23] Cheng HY, Gao Y, Lou G. DNA methylation of the RIZ1 tumor suppressor gene plays an important role in the tumorigenesis of cervical cancer. European Journal of Medical Research. 2010; 15: 20–24.

[24] Yi X, Guo J, Guo J, Sun S, Yang P, Wang J, *et al*. EZH2-mediated epigenetic silencing of TIMP2 promotes ovarian cancer migration and invasion. Scientific Reports. 2017; 7: 3568.

[25] Cao J, Li Z, Yang L, Liu C, Luan X. Association Between Tissue Inhibitor of Metalloproteinase-3 Gene Methylation and Gastric Cancer Risk: A Meta-Analysis. Genetic Testing and Molecular Biomarkers. 2016; 20: 427–431.

[26] Li X, Lu J, Teng W, Zhao C, Ye X. Quantitative Evaluation of MMP-9 and TIMP-1 Promoter Methylation in Chronic Periodontitis. DNA and Cell Biology. 2018; 37: 168–173.

[27] Serioli S, Doglietto F, Fiorindi A, Biroli A, Mattavelli D, Buffoli B, *et al*. Pituitary Adenomas and Invasiveness from Anatomo-Surgical, Radiological, and Histological Perspectives: A Systematic Literature Review. Cancers. 2019; 11: 1936.

[28] Lithgow K, Batra R, Matthews T, Karavitaki N. MANAGEMENT OF ENDOCRINE DISEASE: Visual morbidity in patients with pituitary adenoma. European Journal of Endocrinology. 2019; 181: R185–R197.

[29] Beylerli O, Khasanov D, Gareev I, Valitov E, Sokhatskii A, Wang C, *et al*. Differential non-coding RNAs expression profiles of invasive and non-invasive pituitary adenomas. Non-coding RNA Research. 2021; 6: 115–122.

[30] Kundu S, Das R, Laskar S, Choudhury Y, Ghosh SK. Principles of bi-sulfite conversion of DNA and methylation-specific PCR (MSP) in biological research. Epigenetics Methods. 2020; 17–36.

[31] van Kempen PMW, van Bockel L, Braunius WW, Moelans CB, van Olst M, de Jong R, *et al*. HPV-positive oropharyngeal squamous cell carcinoma is associated with TIMP3 and CADM1 promoter hypermethylation. Cancer Medicine. 2014; 3: 1185–1196.

[32] Maleva Kostovska I, Jakimovska M, Popovska-Jankovic K, Kubelka-Sabit K, Karagjozov M, Plaseska-Karanfilska D. TIMP3 Promoter Methylation Represents an Epigenetic Marker of BRCA1ness Breast Cancer Tumours. Pathology Oncology Research: POR. 2018; 24: 937–940.

[33] Liu HY, Gu WJ, Wang CZ, Ji XJ, Mu YM. Matrix metalloproteinase-9 and -2 and tissue inhibitor of matrix metalloproteinase-2 in invasive pituitary adenomas: A systematic review and meta-analysis of case-control trials. Medicine. 2016; 95: e3904.

[34] Guo H, Sun Z, Wei J, Xiang Y, Qiu L, Guo L, *et al*. Expressions of Matrix Metalloproteinases-9 and Tissue Inhibitor of Metalloproteinase-1 in Pituitary Adenomas and Their Relation-

ships with Prognosis. Cancer Biotherapy & Radiopharmaceuticals. 2019; 34: 1–6.

[35] Mao JH, Guo H, Si N, Qiu L, Guo LF, Sun ZS, *et al*. Regulating effect of MMP-9 and TIMP-1 in pituitary adenoma invasion. Genetics and Molecular Research: GMR. 2015; 14: 17091–17098.

[36] Gonzalez-Avila G, Sommer B, García-Hernández AA, Ramos C. Matrix Metalloproteinases' Role in Tumor Microenvironment. Advances in Experimental Medicine and Biology. 2020; 1245: 97–131.

[37] Stetler-Stevenson WG. The Continuing Saga of Tissue Inhibitor of Metalloproteinase 2: Emerging Roles in Tissue Homeostasis and Cancer Progression. The American Journal of Pathology. 2023; 193: 1336–1352.

[38] Das AM, Bolkestein M, van der Klok T, Oude Ophuis CMC, Vermeulen CE, Rens JAP, *et al*. Tissue inhibitor of metalloproteinase-3 (TIMP3) expression decreases during melanoma progression and inhibits melanoma cell migration. European Journal of Cancer (Oxford, England: 1990). 2016; 66: 34–46.

[39] Tofrizal A, Fujiwara K, Azuma M, Kikuchi M, Jindatip D, Yashiro T, *et al*. Tissue inhibitors of metalloproteinase-expressing cells in human anterior pituitary and pituitary adenoma. Medical Molecular Morphology. 2017; 50: 145–154.

[40] He DS, Chen MZ, Wang HJ, Ke CL, Yan C, Zheng H, *et al*. Role of matrix metalloproteinases-9, 2 and their inhibitor-TIMP-1, 2 in invasive pituitary adenomas biological behavior. Ai Zheng = Aizheng = Chinese Journal of Cancer. 2002; 21: 1124–1128.

[41] Yun J, Park MH, Son DJ, Nam KT, Moon DB, Ju JH, *et al*. IL-32 gamma reduces lung tumor development through upregulation of TIMP-3 overexpression and hypomethylation. Cell Death & Disease. 2018; 9: 306.

[42] Wanga XZ, Gu JL, Gao M, Bian Y, Liang JY, Wen HM, *et al*. Peperomin E Induces Promoter Hypomethylation of Metastatic-Suppressor Genes and Attenuates Metastasis in Poorly Differentiated Gastric Cancer. Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology. 2018; 50: 2341–2364.

[43] Ma HS, Wang EL, Xu WF, Yamada S, Yoshimoto K, Qian ZR, *et al*. Overexpression of DNA (Cytosine-5)-Methyltransferase 1 (DNMT1) And DNA (Cytosine-5)-Methyltransferase 3A (DNMT3A) Is Associated with Aggressive Behavior and Hypermethylation of Tumor Suppressor Genes in Human Pituitary Adenomas. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research. 2018; 24: 4841–4850.