

Demethylation of TIMP2 and TIMP3 inhibit cell proliferation, migration and invasion in pituitary adenoma

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Abstract

Background: Pituitary adenoma (PA) is one of the most common intracranial neoplasms. Tissue inhibitors of metalloproteinases (TIMPs) are prognostic biological markers, but their biological roles remains largely unclear in invasive PA. **Methods:** The promoter methylation status of TIMP2 and TIMP3 genes in invasive PA tissues and cells was measured by methylation-specific polymerase chain reaction (MSP). The expression of TIMP1-3 was validated by quantitative real time PCR and western blot analysis. Overexpression and knockdown of TIMP2 and TIMP3 in GH3 cells were created by transfection of pcDNA3.0 and siRNA against TIMP2 and TIMP3, respectively. Functional experiments in GH3 cells were performed with CCK-8 assay, wound healing assay and transwell assay. Effects of 5-Azacytidene (5-AzaC) on the methylation of TIMP2 and TIMP3 gene, and DNA methyltransferase 1 (DNMT1), DNMT3a and DNMT3b were determined by western blot analysis. **Results:** We found the expression of TIMP1, TIMP2 and TIMP3 was down-regulated in invasive PA tissues and cells. Moreover, decreased TIMP2 and TIMP3 expression was closely associated with their promoter methylation. The *in vitro* experiments showed that overexpression of TIMP2 and TIMP3 exerted suppressive effects, while knockdown of TIMP2 and TIMP3 presented enhanced effects on cell proliferation, migration and invasion in PA cells. Furthermore, 5-AzaC treatment concomitantly up-regulated the protein levels of TIMP2, TIMP3, DNMT1, DNMT3a and DNMT3b. **Conclusions:** In conclusion, our results support that DNA methylation at least partly accounts for TIMP2 or TIMP3 silencing in invasive PA, which will provide new insights into the mechanisms underlying the function of TIMPs in PA. **Key words:** Invasive pituitary adenoma, TIMPs, DNA methylation, 5-AzaC, GH3

Introduction

Pituitary adenoma (PA), accounting for 10 to 15% of intracranial tumors, is one of the most common intracranial neoplasms with an overall prevalence of about 20% in the general population (1-3). Two major symptoms (endocrine-related and tumor occupying symptoms) have been reported to be associated with PA, which could cause some side effects, including mood disorders, sexual dysfunction, obesity, visual disturbances, and diabetes mellitus (4, 5). It was estimated that invasive PA is composed of approximately 30% of PA, which is characterized as tumor invading the adjacent sphenoid sinus and cavernous sinus (5, 6). Since complete resection is difficult for extensive local invasion, better understanding the molecular mechanisms underlying invasive PA is urgently needed for early diagnosis and treatment (7)

Matrix metalloproteinase (MMPs), a family of zinc-containing endopeptidases that can degrade the natural extracellular matrix and basement membrane, allows for the growth and mobility of tumor cells, thereby easily enabling tumor cell proliferation and invasion, including into the PA (8-10). Tissue inhibitors of metalloproteinases (TIMPs) can non-selectively but effectively inhibit the active MMPs (8-10) which have been known to act as tumor suppressor genes in several tumors. TIMP2, a possible antagonist of MMP2, is a metastasis suppressor in the process of invasion of cervical cancer cells (11). TIMP3 is a potential biomarker for predicting the tumor stage and T-status in patients with oral squamous cell

carcinoma (12). Overexpression of TIMP3 suppresses cell proliferation, induces apoptosis, and inhibits migration and invasion in hepatocellular carcinoma (13). Additionally, TIMPs also act as functional tumor suppressors in non-small cell lung cancer (14, 15), prostate cancer (16), and colorectal cancer (17). Notably, our interests were aroused by TIMP1-3 which have been reported to be prognostic biological markers in invasive PA (18-20). However, the exact biological functions of TIMP1-3 and underlying mechanisms in invasive PA remains largely unclear.

In recent times, aberrant DNA methylation by repressing some tumor suppressor genes is the most widely investigated cancer epigenetics. This seems to be a common feature of all human tumors (24-26) Changes of TIMPs expression by methylation have been significant in tumor growth, invasion and metastasis. For example, EZH2 inhibits TIMP2 expression via H3K27me3 and DNA methylation, which relieves the repression of MMP and facilitates ovarian cancer invasion and migration (21). A study by Cao *et al* showed that TIMP3 promoter methylation is positively correlated with gastric cancer risk and could be used as a molecular marker for gastric cancer (22). In addition, female patients exhibited lower methylation levels of MMP-9 but higher methylation levels of TIMP-1 compared to male patients, and the methylation levels of TIMP-1 gradually decreased with age (23). These evidences prompted us to make an assumption that aberrant expression of TIMPs participated in invasive PA cellular function might be associated with transcriptional silence by DNA methylation.

Based on these facts, we first investigated the expression pattern of TIMPs in PA tissues and the methylation status in clinical samples and cell lines. Moreover, we explored the biological function of TIMPs in PA cell proliferation, migration and invasion by RNA interference or 5-AzacytidineAzacytidine treatment. These preliminary results will give us better insights into the effects of TIMPs on progression of invasive PA and help provide new approaches for the prevention and diagnosis of invasive PA.

Materials And Methods

Tissues samples

Human tissue specimens were collected from PA patients, including 20 cases of invasive and 20 cases of non-invasive pituitary tumor. Meanwhile, 20 cases of normal pituitary tissues were obtained from the thin layer of pituitary tissue around the ACTH microadenocarcinoma as control group. All samples were obtained from the Second Affiliated Hospital of Guilin Medical University (Guilin, Guangxi, China). After surgical resection, all the tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Before tissue collection, all patients were administered the informed consent and this study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Guilin Medical University.

Cell culture

Rat PA cell lines, including MMQ, GH1, GH3 and RC-4B/C, and normal pituitary cells were purchased from the American Type Cell Collection (ATCC, Manassas, VA, USA). MMQ, GH1 and GH3 were routinely

cultured in ATCC-formulated F-12K medium (Invitrogen, Carlsbad, CA). RC-4B/C and normal pituitary cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen). All the media were supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/mL penicillin, and 100 units/mL streptomycin. All cell lines were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

Cell treatment and transfection

GH3 cells were used for pharmacological demethylation. Briefly, approximately 1×10^6 cells were seeded in six-well plates and treated with 5-Azacytidine(5-AzaC, Sigma-Aldrich, St. Louis., MO, USA) for 24 at 37°C. For cell transfection, small interfering RNA targeting TIMP2 (siTIMP2), TIMP3 (siTIMP3) and control siRNA (siNC) were chemically synthesized by GenePharma Co. Ltd. (Shanghai, China) and transfected into GH3 cells with a final concentration of 10 nM. Coding sequence of human TIMP2 or TIMP3 was sub-cloned into pcDNA3.0 vector (Invitrogen) to construct TIMP2 or TIMP3 overexpression vector by RiboBio (Guangzhou, China). The plasmid vectors, including pcDNA3.0-TIMP2, pcDNA3.0-TIMP3 or pcDNA3.0 were transfected into the GH3 cells. All cell transfections were performed in accordance with the manual for the reagent transfection Lipofectamine 2000 (Invitrogen). After 48 h transfection, the cells were harvested for the subsequent experiments.

Quantitative real time PCR

Total RNA was extracted from tissue samples or cell lines using TRIzol reagent (Invitrogen) and reverse transcribed using M-MLV reverse transcriptase (Bio-Rad, Minneapolis, MN, USA) by extension of oligo primers (TaKaRa). Quantitative real time PCR was performed with DBI Bestar[®] SybrGreen *qPCR Master Mix* on a Stratagene Real time PCR (Mx3000P, Agilent). The mRNA expression was calculated by using the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of GAPDH mRNA. The primer sequences are shown in **Table 1**.

Western blot analysis

Total protein was extracted from tissue samples or cell lines using RIPA lysis buffer (Roche, Complete Mini). The protein concentration was determined using bicinchoninic acid method. Approximately 30 µg of the total protein was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting analysis and electro-transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories Inc., USA). After blocking with Tris-buffered saline containing 0.1% Tween 20 (TBST, Sigma, USA), the membranes were incubated with primary antibodies against TIMP1 (1:1000), TIMP2 (1:2000), TIMP3 (1:4000), DNMT1 (1:1000), DNMT3a (1:2000), DNMT3b (1:1500) and GAPDH (1:10000) all from Abcam (Cambridge, UK) at 4°C overnight. On the next day, the membranes were washed with TBST three times (5 min each time), followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:20000, BA1051, BA1054) for 2 h at room temperature. Subsequently, the protein signals were detected using enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Methylation-specific PCR (MSP)

Genomic DNA was extracted from tissues or cells using High Pure PCR Template Preparation Kit (Roche Applied Science, Pennsburg, Germany). The sequence of promoter 5'-C-phosphate-G-3' (CpG) islands of the relevant genes was predicted online (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>). The DNA samples were treated with sodium bisulfite using the EpiTect Bisulfite Kit (QiaGen, Hilden, Germany), followed by DNA modification by EZ DNA Methylation-Gold Kit (Zymo Research). Bisulfite DNA was amplified with methylated or unmethylated specific primer sequences designed by Methyl primer Express v1.0, as listed in **Table 2**. MSP was used to amplify methylated gene and MSP products were analyzed by 2% agarose gel electrophoresis. Methylated and non-methylated human DNAs were used as positive and negative controls, respectively.

CCK-8 assay

Treated or transfected GH3 cells at a density of 3,000 cells per well were seeded in a 96-well plate and incubated overnight at 37°C. Then, cell proliferation was determined using Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, 10 µL CCK-8 solutions was added to each well at 24, 48, and 72 h, respectively, followed by incubation for 2 h at 37°C. The optical density values were measured using a microplate reader (Bio-Tek, USA) at 450 nm.

Wound healing assay

Wound healing assay was performed to evaluate cell migration ability. Briefly, GH3 cells at a density of 4,000 cells per well were seeded in six-well plates and cultured until 90% confluence. Subsequently, a 100-µL sterile pipette tip was used to artificially create a straight scratch through the monolayer. The images of the same location at 0 and 48 h after wounding in five randomly selected fields were observed under a microscope (Olympus, Tokyo, Japan). The relative average migration distance was calculated as follows: $(\text{Width}_{0\text{h}} - \text{Width}_{48\text{h}}) / \text{Width}_{0\text{h}} \times 100\%$.

Cell invasion assay

For cell invasion assay, approximately 3×10^5 cells in serum free medium were added to the upper chamber coated with Matrigel (Millipore) in 24-well transwell insert. Then, 500 µL serum-containing medium was added to the lower chamber. After 48 h incubation, cells that had invaded to the lower chamber were fixed and stained with 0.5 % crystal violet. Stained cells were photographed and counted in five randomly selected fields under a light microscope.

Statistical analysis

Quantitative data were analyzed with SPSS 21.0 software package and expressed as mean \pm standard deviation (SD) of at least three repetitive experiments. Comparisons of continuous data were performed using two-tailed t-test for two groups or one-way analysis of variance for multiple comparisons data. The values of *p* less than 0.05 were considered to be statistically significant differences.

Results

The expression and methylation status of TIMP1-3 in PA tissues

Quantitative real time PCR was first performed to determine the expression of TIMP1, TIMP2, and TIMP3 in human PA samples, including 20 cases of invasive, 20 cases of non-invasive pituitary tumor and 20 matched controls. As shown in **Figure 1A**, the results showed that the expression of TIMP1-3 was down-regulated in invasive pituitary tumor and non-invasive pituitary tumor compared with matched controls. Notably, the expression of TIMP1-3 was obviously decreased in invasive pituitary tumor compared with non-invasive pituitary tumor, indicating their associated with tumor metastasis. Representative three pairs of tissue samples were applied to the western blot analysis. Consistently, all the protein levels of TIMP1-3 were presented the same change trend as results from quantitative real time PCR (**Figure 1B**). To better explain the aberrant expression of TIMP1-3 in PA tissues, the methylation status of CpG sites of TIMP1-3 promoter was predicted online. The predicted results showed only TIMP2 and TIMP3 contained typical CpG islands, which indicated the potential role of promoter CpG methylation in TIMP2 and TIMP3 silencing. Next, we focus the methylation status of TIMP2 and TIMP3 by quantifying the degree of their methylation. MSP assay showed that the methylation percentage of TIMP2 (**Figure 1C**) and TIMP3 (**Figure 1D**) in representative three cases of invasive pituitary tumors was 100%, but 0% in non-invasive tumors and matched controls. Taken together, both TIMP2 and TIMP3 expression were decreased, which might be associated with their promoter methylation in invasive pituitary tumors.

Both TIMP2 and TIMP3 exerted suppressive effects on cell proliferation, migration and invasion in PA cells

To further investigate the biological function of TIMP2 and TIMP3, their expression levels were first determined in several PA cell lines by quantitative real time PCR and western blotting. The results revealed that the expression of TIMP2 and TIMP3 mRNA (**Figure 2A-B**) and protein (**Figure 2C**) levels were significantly down-regulated in PA cell lines (MMQ, GH1, GH3 and RC-4B/C), in comparison with normal rat pituitary cells. It is noteworthy that TIMP2 and TIMP3 expression was the lowest in GH3 cells among all the PA cell lines. Subsequently, GH3 cells were transfected with siTIMP2, siTIMP3 or pcDNA3.0-TIMP2, pcDNA3.0-TIMP3 to construct TIMP2 and TIMP3 silenced or overexpressed cells. As illustrated in quantitative real time PCR, the mRNA expression of TIMP2 and TIMP3 were both significantly reduced after the corresponding siRNA transfection, but elevated after pcDNA3.0 transfection in GH3 cells (**Figure 2D**). Consistently, western blotting showed the same expression levels (**Figure 2E**). A series of functional experiments, including CCK-8 (**Figure 2F**), wound healing (**Figure 3A**) and transwell invasion assays (**Figure 3B**) showed that knockdown of TIMP2 or TIMP3 significantly promoted cell proliferation, migration and invasion. Meanwhile, overexpression of TIMP2 or TIMP3 remarkably suppressed cell proliferation, migration and invasion in GH3 cells.

Demethylation of TIMP2 or TIMP3 inhibited cell proliferation, migration and invasion in PA cells

To analyze whether promoter methylation affected the expression of TIMP2 and TIMP3 *in vitro*, GH3 cells were treated with 5-AzaC. As demonstrated by MSP, 5-AzaC treatment obviously reduced the methylation status of TIMP2 and TIMP3 in GH3 cells (**Figure 4A**). As expected, 5-AzaC treatment significantly upregulated the expression of TIMP2 ($p < 0.001$) and TIMP3 ($p < 0.01$) mRNA levels (**Figure 4B**) and protein levels (**Figure 4C**). In addition, western blot demonstrated that 5-AzaC treatment repressed the expression of DNMT1, DNMT3a and DNMT3b (**Figure 4C**). Subsequently, we investigated whether demethylation of TIMP2 or TIMP3 by 5-AzaC treatment presented similar results with TIMP2 or TIMP3 overexpression. The *in vitro* functional assay showed 5-AzaC treatment significantly inhibited cell proliferation, migration and invasion, as determined by CCK-8 assay (**Figure 4D**), wound healing (**Figure 4E**) and transwell invasion assays (**Figure 4F**). Collectively, these results demonstrated that ectopic expression of TIMP2 and TIMP3 by demethylation also exerted tumor suppressive effects on PA cell proliferation, migration and invasion.

Discussion

In the present study, we evaluated the expression patterns of TIMP1-3 in patients with PA using quantitative real time PCR and demonstrated that TIMP1-3 was significantly down-regulated in invasive PA tissues. Moreover, we first revealed that downregulation of TIMP2 or TIMP3 was partly due to the promoter DNA methylation status in invasive PA tissues. Similarly, demethylation could reduce methylation of the TIMP2 promoter and finally reactivate TIMP2 transcription to promote ovarian cancer invasion and migration (21). TIMP3 hypermethylation was found in HPV-positive oropharyngeal squamous cell carcinoma (24). A recent study by Maleva Kostovska *et al.* showed that TIMP3 promoter methylation is an epigenetic marker of BRCA1 positive tumors (25). We focused on the role and methylation status of TIMP2 and TIMP3 in the subsequent experiments because only TIMP2 and TIMP3 contained typical CpG islands in PA tissues.

The *in vitro* functional assays demonstrated that PA cell proliferation, migration, and invasion were significantly enhanced by knockdown TIMP2 or TIMP3, but remarkably suppressed by TIMP2 or TIMP3 overexpression. TIMPs are endogenous regulators of MMPs by inhibiting MMP activity, which are down-regulated in many solid tumors, and act as repressors of tumor metastasis (26, 27). For instance, TIMP3 functions as a tumor suppressor in melanoma and negatively regulates several aspects of the metastatic cascade (28). Furthermore, TIMPs are important in the maintenance of extracellular matrix in the human pituitary (29). Invasive PA less significantly expresses TIMP-1 and TIMP-2 (36)

Invasive PA were significantly less expressing TIMP-1 and TIMP-2 (30). Therefore, we speculated that TIMP2 and TIMP3 might act as tumor suppressors in invasive PA cells.

For further confirmation, decreased TIMP2 or TIMP3 levels were correlated with DNA methylation, we used an agent of DNA demethylation, 5-AzaC to treat GH3 cells and analyzed its effects on cell proliferation, migration, and invasion. Consistent with TIMP2 or TIMP3 overexpression, 5-AzaC

significantly suppressed GH3 cell proliferation, migration, and invasion. We also showed that the expression of DNMT1, DNMT3a and DNMT3b was decreased in 5-AzaC treated GH3 cells accompanied increased TIMP2 and TIMP3. It has been previously reported that inhibition of promoter hypermethylation could cause a marked increase in TIMP3 expression via decreased DNMT1 expression in lung cancer (31). Except for 5-AzaC, a natural secolignan, peperomin E also exhibits potential as a DNA methylation inhibitor to down-regulates DNMT1, 3a, and 3b expression and induces metastatic-suppressor genes, including E-cadherin and TIMP3 promoter hypomethylation in gastric cancer cells(32). Ma *et al.* have support a possible role of DNMT1 and DNMT3a in tumor suppressor gene promoter methylation leading to PA invasion and suggest that inhibition of DNMTs has the potential to become a new therapeutic approach against invasive PA (33). These data further suggest that DNA methylation might be a major cause for downregulation of TIMP2 or TIMP3 and that the inhibition of promoter hypermethylation could effectively promote the tumor suppressive effects of TIMPs in PA cells.

In summary, this study, for the first time, described that the loss of TIMP2 or TIMP3 in invasive PA is partly due to epigenetic hypermethylation at the promoter regions. Restored TIMP2 or TIMP3 by RNA interference or 5-AzaC treatment could promote its suppressive role in PA cell proliferation, migration, and invasion. These findings will provide new insights into the mechanisms underlying the function of TIMPs in PA.

Declarations

Ethics approval and consent to participate

Before tissue collection, all patients were administered the informed consent and this study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Guilin Medical University.

Consent to publish

Not applicable

Availability of data and materials

The data used in this research are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding

Not applicable

Author's contribution

SL conceived and designed research, drafted and revised manuscript; YY, FH, XW and CH performed experiments; FH and XW analyzed data and prepared figures; All authors approved final version of manuscript;YY edited and revised manuscript.

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Not applicable

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Tables

Table 1 Primers used for quantitative real time PCR analysis

Gene	Primer sequence 5'-3'	PS (bp)
TIMP1	F: AGAGTGTCTGCGGATACTTCC R: CCAACAGTGTAGGTCTTGGTG	169
TIMP2	F: AAGCGGTCAGTGAGAAGGAAG R: GGGGCCGTGTAGATAAACTCTAT	136
TIMP3	F: CAGGTCGCGTCTATGATGGC R: AGGTGATACCGATAGTTCAGCC	109
GAPDH	F: TGTTTCGTCATGGGTGTGAAC R: ATGGCATGGACTGTGGTCAT	154

F: forward; R: reverse; PS: product size

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

Gene	Primer sequence 5'-3'	PS (bp)
TIMP2	M-F: ATTATAGGTATTAGATGGGTTGCGA M-R: TCGAAAACTCCTACTTAAAAACG	214
TIMP2	U-F: ATTATAGGTATTAGATGGGTTGTGA U-R: CAAAAACTCCTACTTAAAAACACC	213
TIMP3	M-F: GATGTATACGGGTTGTGTAATTTTC M-R: ATATCGATCCAAAACACTCGTT	168
TIMP3	U-F: TGTATATGGGTTGTGTAATTTTGT U-R: ACATATCAATCCAAAACACTCATT	168

M-F: methylated forward; M-R: methylated reverse; U-F: un-methylated forward; U-R: un-methylated reverse; PS: product size

Figures

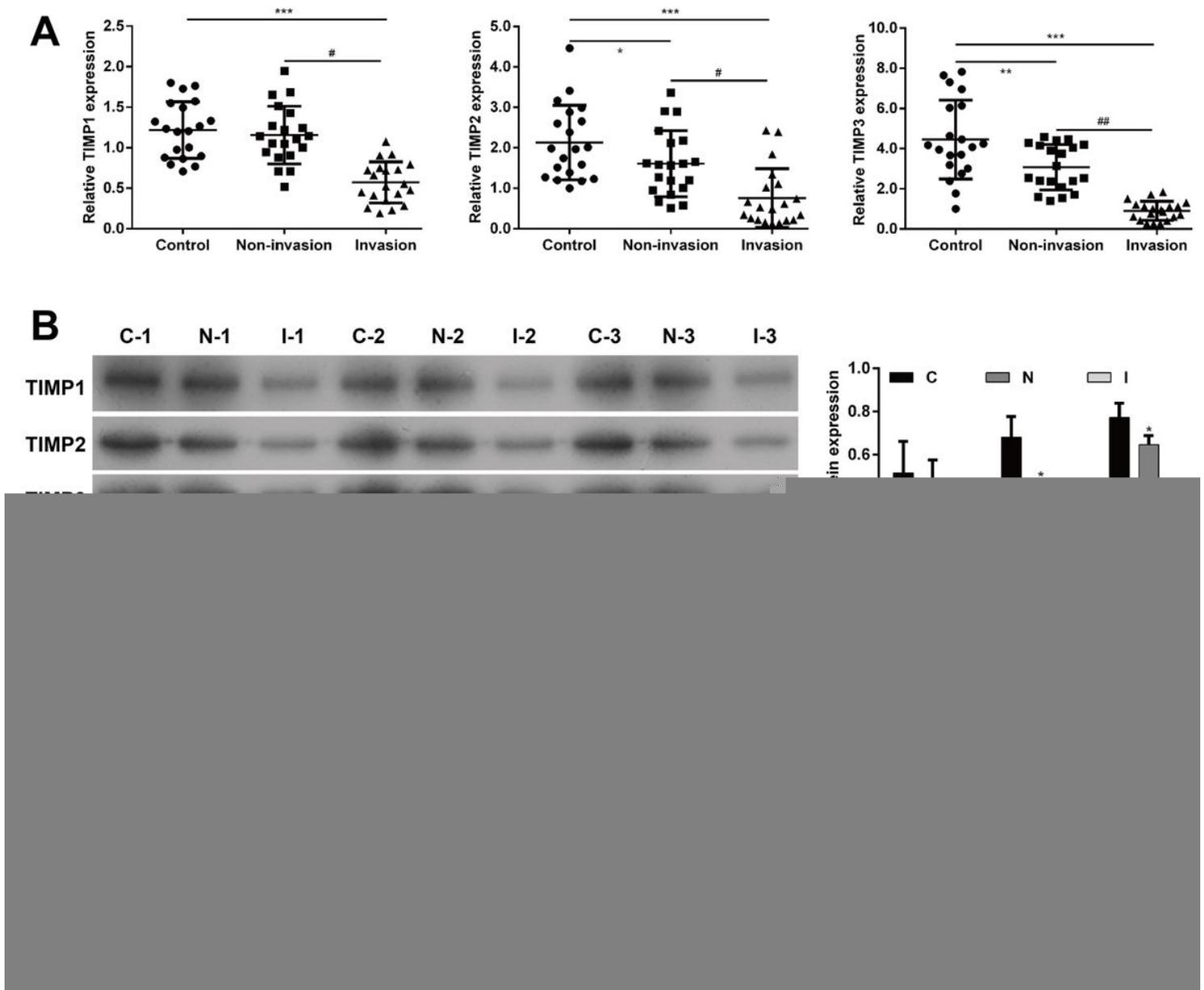


Figure 1

Analysis of TIMP2 and TIMP3 expression and methylation status in pituitary adenoma tissues; (A) Quantitative real time PCR was used to determine the expression of TIMP1-3 mRNA levels in 20 cases of invasive, 20 cases of non-invasive pituitary tumor and 20 matched controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with Control; # $p < 0.05$, ## $p < 0.01$, compared with non-invasion; (B) The protein levels of TIMP1-3 were detected in representative three pairs of tissue samples. * $p < 0.05$, *** $p < 0.001$, compared with C; ## $p < 0.01$, compared with N; GAPDH was used as an internal control. The methylation status of (C) TIMP2 and (D) TIMP3 genes was measured in representative three pairs of tissue samples via MSP assay. +, positive control; -, negative control; Abbreviations: C, control; N, non-invasive; I, invasive; M, methylated primer; U, un-methylated primer

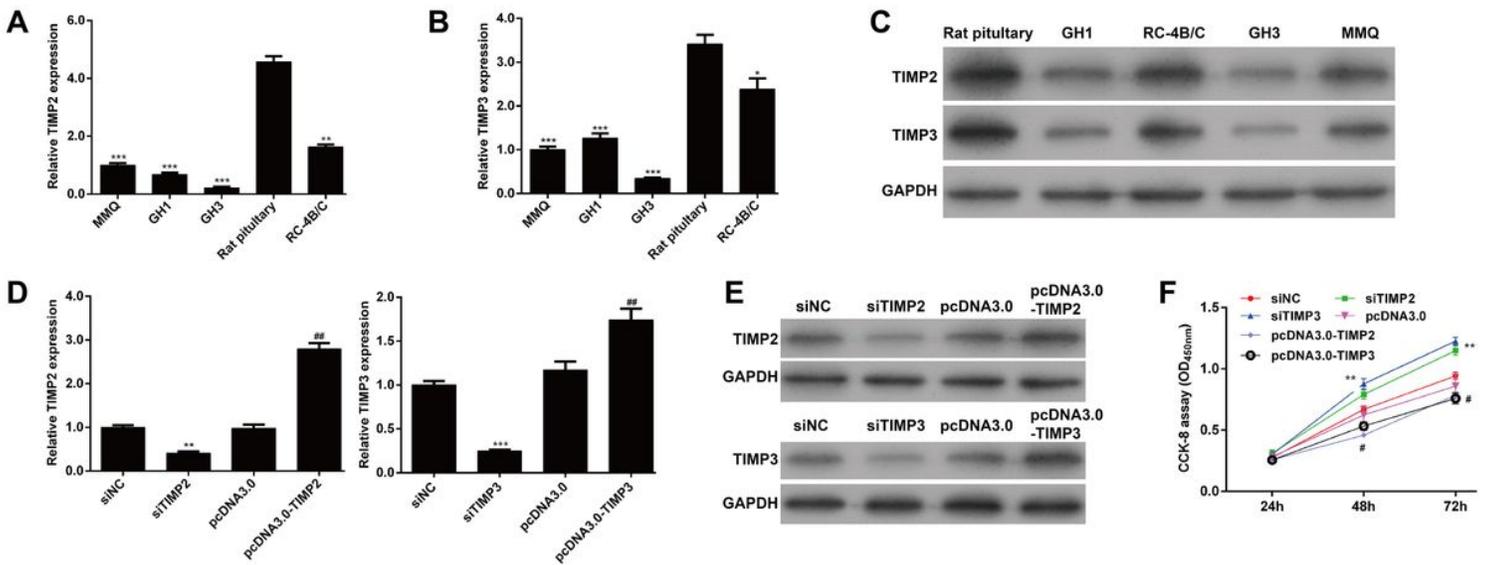


Figure 2

TIMP2 and TIMP3 suppressed cell proliferation in pituitary adenoma cells. (A-B) Quantitative real time PCR and (C) western blot were used to determine the expression levels of TIMP2 and TIMP3 in pituitary adenoma cell lines, including MMQ, GH1, GH3 and RC-4B/C, as well as normal rat pituitary cells. Data were expressed as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with rat pituitary cells; GH3 cells were transfected with siNC, siTIMP2, siTIMP3, pcDNA3.0, pcDNA3.0-TIMP2 and pcDNA3.0-TIMP3, respectively. (D) The expression of TIMP2 and TIMP3 mRNA levels were determined using quantitative real time PCR. (E) Western blot was used to measure the protein levels of TIMP2 and TIMP3 in GH3 cells. (F) Cell proliferation ability was assessed by CCK-8 assay. Data were expressed as mean \pm SD. ** $p < 0.01$, *** $p < 0.001$, compared with siNC; # $p < 0.05$, ## $p < 0.01$, compared with pcDNA3.0

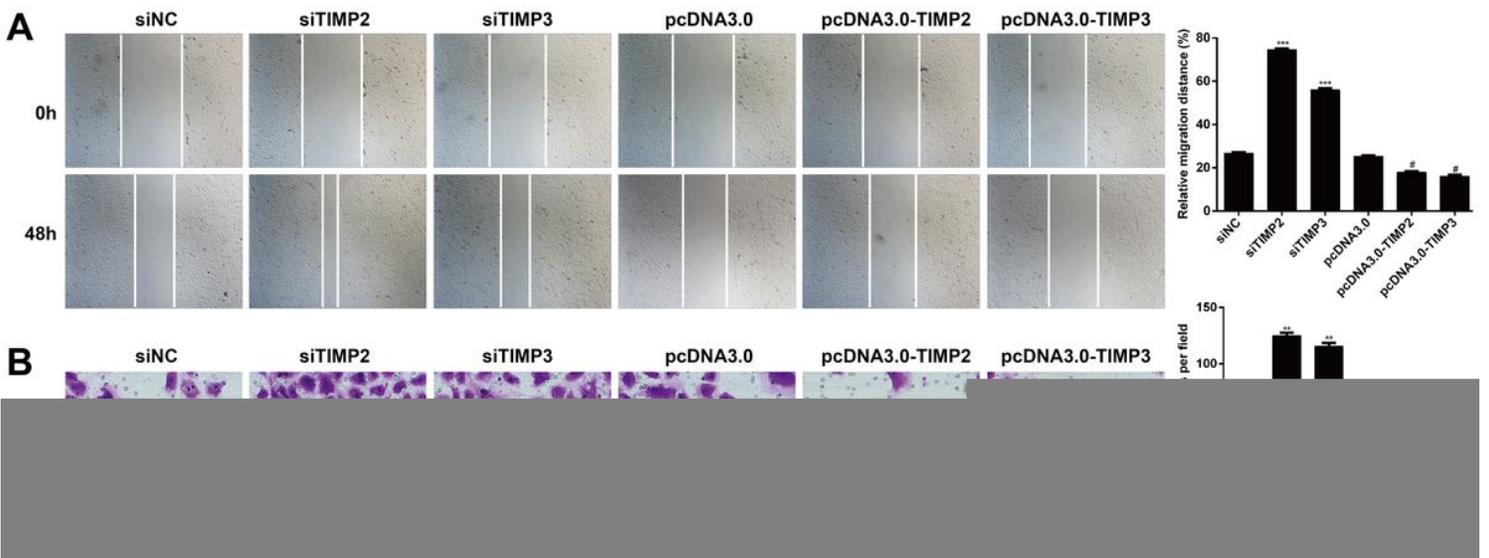


Figure 3

TIMP2 and TIMP3 suppressed cell migration and invasion in pituitary adenoma cells. GH3 cells were transfected with siNC, siTIMP2, siTIMP3, pcDNA3.0, pcDNA3.0-TIMP2 and pcDNA3.0-TIMP3, respectively.

(A) The cell images (magnification, 40×) of wound-healing assay (left panel). The relative cell migrated area at 48 h compared with time 0 h was measured using Image J software in GH3 cells (right panel). (B) The images (magnification, 200×) of cells that migrated into plate wells (lower chamber) in the transwell invasion assay in GH3 cells (left panel). The number of migrated cells was counted by Image J software (right panel). Data were expressed as mean ± SD. ** $p < 0.01$, *** $p < 0.001$, compared with siNC; # $p < 0.05$, ## $p < 0.01$, compared with pcDNA3.0

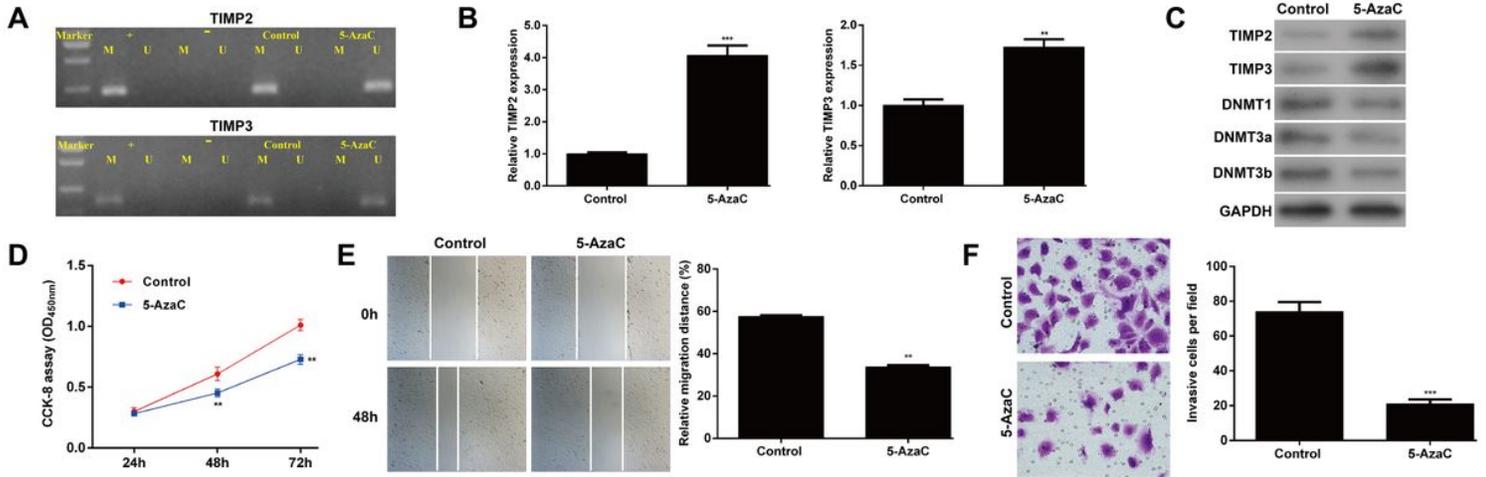


Figure 4

Ectopic expression of TIMP2 and TIMP3 by demethylation suppressed cell proliferation, migration and invasion. (A) MSP analysis of TIMP2 and TIMP3 in GH3 cells after treatment with 5-AzaC; Abbreviations: +, positive control; -, negative control; M, methylated primer; U, un-methylated primer; (B) The expression of TIMP2 and TIMP3 mRNA levels were determined using quantitative real time PCR. (C) Western blotting was used to detect the expression of TIMP2, TIMP3, DNMT1, DNMT3a and DNMT3b in GH3 cells after treatment with 5-AzaC. (D) CCK-8 assay was used to determine cell proliferation in GH3 cells after 5-AzaC treatment. (E) Wound-healing assay and (F) transwell invasion assay were performed to evaluate cell migration and invasion ability. Data were expressed as mean ± SD. ** $p < 0.01$, *** $p < 0.001$, compared with control.