

Suppression of PDCD4 mediated by the long non-coding RNA HOTAIR inhibits the proliferation and invasion of glioma cells

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Received April 26, 2015; Accepted May 26, 2016

DOI: 10.3892/ol.2016.5323

Abstract. Programmed cell death protein 4 (PDCD4) has recently been demonstrated to be implicated in translation and transcription, and the regulation of cell growth. However, the mechanisms underlying PDCD4 function in glioma cells remain to be elucidated. The current study investigated the function and regulation of PDCD4 and the results demonstrated that the expression of PDCD4 was significantly reduced in glioma cells compared with normal cells. When PDCD4 was overexpressed in glioma cells, the proliferation rate and invasive capability of the cells greatly decreased, suggesting that PDCD4 functions as a tumor suppressor in this cell type. In addition, the histone modification status of the PDCD4 gene was analyzed, and chromatin immunoprecipitation assay identified a high density of histone 3 lysine 27 trimethylation on the promoter of PDCD4, which was associated with the long non-coding RNA, homeobox transcript antisense RNA (HOTAIR). The expression of HOTAIR was significantly increased in glioma cells compared with normal cells, and it exerted its function in a polycomb repressive complex 2-dependent manner. These results may provide novel approaches to therapeutically target PDCD4 and HOTAIR in patients with gliomas.

Introduction

Human gliomas are the most prevalent malignant neoplasms of the central nervous system, with an annual incidence of ~5/100,000 worldwide (1). Despite the use of aggressive surgery in combination with chemotherapy, biological therapy and radiotherapy, gliomas continue to be therapeutically

challenging (2). For patients with glioblastoma, the relative 5-year survival rate is <5% (3). Novel therapies for the treatment of glioma are warranted; recent advances in the understanding of the molecular and biological nature of this disease may facilitate the development of successful therapeutics (4).

PDCD4 protein was initially determined to be overexpressed during apoptosis, which subsequently suppresses tumorigenesis (5,6). Loss of PDCD4 expression is closely associated with the progression of a number of tumors, including glioblastomas (7), and kidney, ovarian and lung cancer (8-10). Low PDCD4 expression levels correlate with poor outcomes in patients with glioblastoma multiforme (11). The frequent loss of PDCD4 in glioblastoma multiforme is partly due to epigenetic silencing secondary to 5' cytosine-phosphate-guanine island methylation (12), in addition to overexpression of microRNA (miRNA)-21, which targets PDCD4 mRNA for degradation (13). Although several studies have examined PDCD4 in glioma, the detailed molecular mechanisms underlying the role of PDCD4 in glioma remain poorly understood.

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts longer than 200 nucleotides, which are involved in various important events, including transcriptional, epigenetic and post-transcriptional regulation (14,15). A previous study profiled the lncRNA homeobox transcript antisense RNA (HOTAIR), and the results demonstrated that HOTAIR was closely correlated with poor prognosis, molecular subtype and tumor grade in patients with glioma (16). However, the details of how HOTAIR regulates tumor suppressors, including PDCD4, remain unclear.

The results of the present study demonstrated that PDCD4 functions as a tumor suppressor in glioma cells, and its downregulation is associated with a high level of histone 3 lysine 27 trimethylation (H3K27me3) at the PDCD4 promoter, a level that is mediated by HOTAIR in a polycomb repressive complex 2 (PRC2)-dependent manner.

Materials and methods

Experimental subjects. A total of 24 brain glioma tissue samples and their matched adjacent normal tissues from 24 patients obtained following surgical resection were collected from the Department of Neurosurgery, Yantai Yuhuangding Hospital Affiliated to Qingdao University Medical College (Yantai, China) between August 2010 and September 2012. Adjacent

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Key words: programmed cell death protein 4, long non-coding RNA, homeobox transcript antisense RNA, glioma

tissues were located 1 cm away from lesions. All specimens were obtained under sterile conditions during surgery, and immediately placed into Eppendorf tubes and frozen at -80°C . The present study was approved by the ethics committee of Shandong University (Jinan, China; approval no. 20130041). Written informed consent was obtained from all patients.

Cell preparation and culture. The human astrocyte HA cell line was purchased from ScienCell Research Laboratories (San Diego, CA, USA). The human glioma cell lines U251, U87, LN-18 and H4 were all purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with heat-inactivated 10% fetal calf serum (GE Healthcare Life Sciences), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 .

Cell transfection and RNA interference. The lentivirus for PDCD4 overexpression (Lenti-PDCD4) and the control (Lenti-Empty) were commercially constructed by Genechem Co., Ltd. (Shanghai, China). The lentivirus was packaged in HEK-293T cells and collected from the supernatant following the manufacturer's protocol. Glioma cells were infected with lentiviral particles. Cell lines stably expressing PDCD4 were established using puromycin as the selection marker.

Small interfering RNA (siRNA) targeting HOTAIR was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Cells were transfected using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). RNA was isolated from the human glioma cell lines using RNAzol[®] reagent (Vigorous Biotechnology Co., Ltd., Beijing, China), according to the manufacturer's protocol. The RNA was treated with DNase H (Beyotime Institute of Biotechnology, Haimen, China) to remove contaminating genomic DNA. cDNA was synthesized in a 25 μl reaction mixture consisting of 2 μg total RNA, 1 μl M-MLV Reverse Transcriptase 2 μl dNTPs, 5 μl 5X buffer, 1 μl random primers, 0.5 μl RNasin and diethylpyrocarbonate (all Promega Corporation, Madison, WI, USA). qPCR was performed using the ABI 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with reagents from the SYBR[®] Green Real-Time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) and the appropriate primers, which are presented in Table I. The PCR cycling conditions were as follows: 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Relative mRNA expression levels were determined following normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta\text{C}_q}$ method (17).

Cell proliferation. Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to determine cell proliferation rate according to the manufacturer's protocol at the indicated time points. Briefly, cells

were seeded in 96-well plates at a density of 2,000 cells/well. Cell proliferation reagent (10 μl) was added to each well, and the cells were incubated for 2 h at 37°C . Cell numbers were estimated by measuring the optical density at 450 nm. The absorbance of cell-free wells containing medium was set as zero. Data was obtained from three separate experiments and three replications were performed each time.

Cell invasion assay. Transwell chambers (8.0 μm pore size; Corning Incorporated, Corning, NY, USA) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used to measure the invasiveness of glioma cells. In brief, 5×10^4 cells/well were seeded in the upper chamber in DMEM without serum, and the lower chamber contained DMEM supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) to stimulate cell invasion. Following 48 h of incubation at 37°C , cells that migrated to the bottom of the chamber insert were fixed with 3% paraformaldehyde, stained with 0.1% crystal violet, extracted with 33% acetic acid and finally detected quantitatively using a standard microplate reader (at 570 nm). Data was obtained from three separate experiments and three replications were performed each time.

Western blot analysis. Protein extracts (10 μg) prepared with radioimmunoprecipitation assay buffer were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. Subsequent to blocking with 5% non-fat milk, the membranes were incubated overnight at 4°C with mouse anti-PDCD4 (#sc-376430) and mouse anti-GAPDH (#sc-25778) monoclonal antibodies (dilutions, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Blots were then incubated with peroxidase-conjugated goat anti-mouse secondary antibodies (dilution, 1:1,000; #ZB2305 and #ZB2307; Beijing Zhongshan Jinqiao Biotechnology, Co., Ltd., Beijing, China) for 1 h at room temperature and developed using a SuperSignal[™] West Pico Chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL, USA). Immunoblots were scanned using Image Lab[™] software, version 1709690 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Chromatin immunoprecipitation (ChIP) assay. Cells were fixed with 10% formaldehyde and sonicated to prepare the chromatin sample. Chromatin samples were immunoprecipitated with mouse anti-H3K27me3 monoclonal antibody (#ab6002; Abcam, Cambridge, MA, USA), rabbit anti-enhancer of zeste homolog 2 (EZH2) polyclonal antibody (#ab3748; Abcam) or rabbit immunoglobulin G (IgG; #ab6785; Abcam) at 4°C for 3 h. Following crosslink reversal, precipitated DNA was analyzed by PCR for fragments of the PDCD4 promoter using the following primers: Forward, 5'-GGGAGGAGGAATCGGACAG-3'; and reverse, 5'-TATGTTGGGAGCGTGGC-3' (141 bp). The PCR cycling conditions were as follows: 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. The data obtained were normalized to those of corresponding DNA precipitated by IgG.

Statistical analysis. All data are expressed as the mean \pm standard deviation. Comparisons between two groups were performed using Student's *t*-test or among groups

Table I. Primer sequences for each gene.

Gene	Primer sequences, 5'-3'	Product size, bp
GAPDH		116
Forward	TGTGGGCATCAATGGATTTGG	
Reverse	ACACCATGTATTCCGGGTCAAT	
PDCD4		108
Forward	GGGAGTGACGCCCTTAGAAG	
Reverse	ACCTTTCTTTGGTAGTCCCCTT	
HOTAIR		135
Forward	GGCAGCACAGAGCAACTCTA	
Reverse	GAGTGCAAAGTCCCCTTTG	

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PDCD4, programmed cell death protein 4; HOTAIR, homeobox transcript antisense RNA.

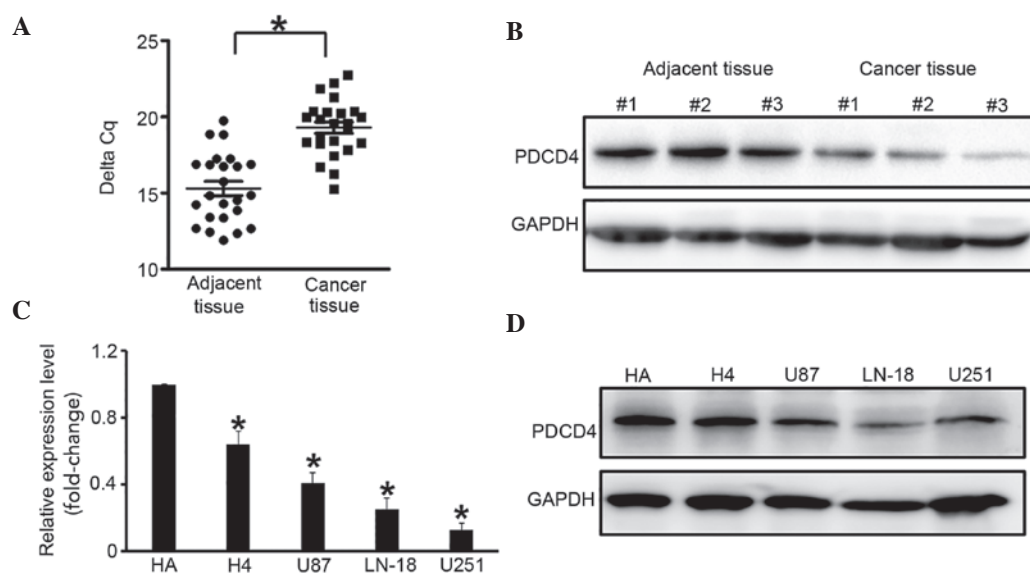


Figure 1. PDCD4 expression is downregulated in glioma cells. (A) Δ Cq values for PDCD4 RNA levels in 24 glioma tissue samples. (B) PDCD4 protein levels in 3 representative glioma tissue samples. (C) Relative PDCD4 RNA levels in the HA, H4, U87, LN-18 and U251 cells. Data represents the results of three independent experiments. (D) PDCD4 protein levels in the HA, H4, U87, LN-18 and U251 cells. * $P < 0.05$ vs. negative control. PDCD4, programmed cell death protein 4; Cq, quantification cycle; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

with one-way analysis of variance. Statistical analyses were conducted using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PDCD4 expression is downregulated in glioma cells. Human glioma tissue samples ($n=24$) were analyzed to detect the change in expression of PDCD4 in glioma. RT-qPCR demonstrated that PDCD4 was significantly downregulated in glioma tissues (by ~20%), as compared with adjacent normal tissues ($P=0.034$; Fig. 1A), which was supported by western blot analysis (Fig. 1B). In a few samples, no PDCD4 expression was detected.

This experiment was repeated with glioma cell lines (U251, LN-18, U87 and H4), and the human astrocyte HA cell line was used as the control. RT-qPCR and western blot analysis demonstrated that PDCD4 expression was suppressed in all

glioma cell lines compared with the HA cells, and its expression was the lowest in the U251 cells (Fig. 1C and D).

PDCD4 inhibits cell growth and invasion in glioma cells. Next, the potential functions of PDCD4 in glioma were investigated. A lentivirus was constructed, Lenti-PDCD4, containing the full length PDCD4 cDNA, and PDCD4 was overexpressed in U251 cells by infection with Lenti-PDCD4. Western blot analysis indicated that PDCD4 was successfully overexpressed compared with the Lenti-Empty construct (Fig. 2A). CCK-8 assay was employed to determine whether PDCD4 affects the proliferation of glioma cells. Glioma cells infected with Lenti-PDCD4 exhibited a significantly lower proliferation rate than the control at 48 and 60 h following transfection ($P < 0.05$; Fig. 2B). In addition, Transwell migration assays were performed to verify invasive ability. The results demonstrated that PDCD4 overexpression resulted in a significant decrease in the invasion rate of U251 cells compared with the control ($P < 0.05$; Fig. 2C).

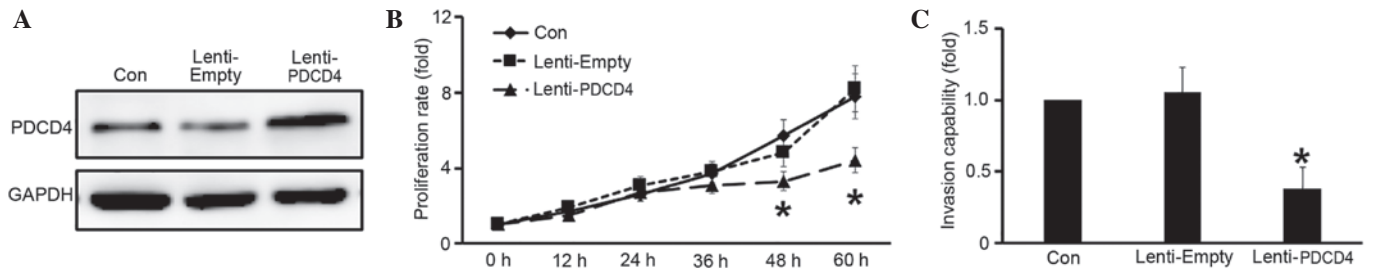


Figure 2. PDCD4 inhibits cell growth and invasion in glioma cells. (A) Expression level of PDCD4 in U251 cells infected with lentivirus overexpressing PDCD4 (Lenti-PDCD4). Lentivirus containing the empty vector (Lenti-Empty) was used as a negative control. (B) Cell counting kit-8 assay was performed on U251 cells overexpressing PDCD4. (C) Transwell analysis was performed to determine invasive capability and luciferase detection values for each cell type were compared. All experiments were repeated three times. * $P < 0.05$ vs. negative control. PDCD4, programmed cell death protein 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Con, control.

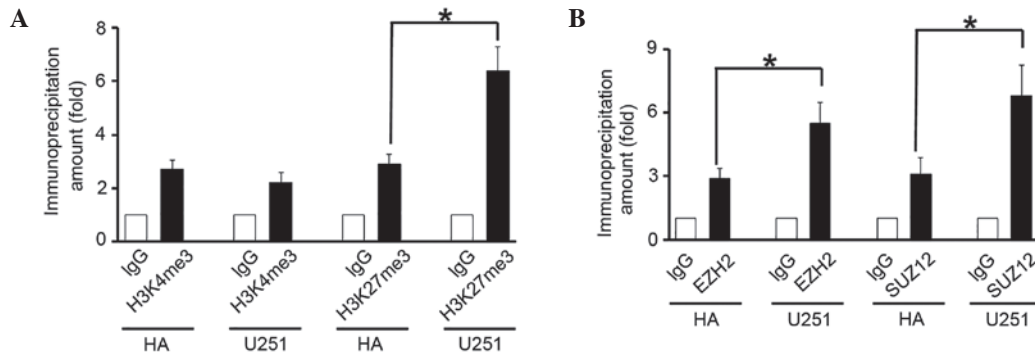


Figure 3. Histones of the PDCD4 promoter may be methylated by the polycomb repressive complex 2 complex. (A) ChIP analysis of H3K4me3 and H3K27me3 histone modification at the PDCD4 promoter in HA and U251 cells. (B) ChIP analysis of EZH2 and SUZ12 recruitment to the PDCD4 promoter in HA and U251 cells. All experiments were repeated three times. * $P < 0.05$ vs. negative control. PDCD4, programmed cell death protein 4; ChIP, chromatin immunoprecipitation; IgG, immunoglobulin G; H3K4me3, histone 3 lysine 4 trimethylation; H3K27me3, histone 3 lysine 27 trimethylation; EZH2, enhancer of zeste homolog 2; SUZ12, suppressor of zeste 12.

Histones at the PDCD4 promoter may be methylated by the PRC2 complex. Next, mechanisms underlying PDCD4 downregulation in glioma were investigated. Epigenetic modifications, particularly methylation at specific histone sites, are important in gene expression. ChIP assays demonstrated that the level of H3K27me3 at the PDCD4 promoter region increased significantly in the U251 cells, as compared with the HA cells ($P = 0.019$; Fig. 3A), thus favoring transcriptional silencing. Conversely, H3K4me3 exhibited little change between the HA and U251 cells ($P > 0.05$; Fig. 3A). The levels of EZH2 and suppressor of zeste 12 (SUZ12), core components of the PRC2 complex, at the PDCD4 promoter region were significantly increased in the U251 cells, as compared with the HA cells ($P < 0.05$; Fig. 3B). These results indicated that the PRC2 complex was able to downregulate PDCD4 expression by increasing the level of H3K27me3 at its promoter.

HOTAIR is upregulated in glioma cells. The expression of HOTAIR was measured in human glioma tissue samples. RT-qPCR demonstrated that HOTAIR expression was significantly elevated (by ~30-fold) in glioma tissues, as compared with normal tissues ($P = 0.039$; Fig. 4A). A similar trend was observed in the glioma cell lines, in which HOTAIR RNA levels were significantly elevated compared with the HA cells ($P < 0.05$; Fig. 4B). To investigate the function of HOTAIR, the gene was knocked down in U251 cells using siRNA, which

significantly decreased its expression, as compared with the scramble RNA ($P < 0.05$; Fig. 4C).

HOTAIR participates in the silencing of PDCD4 in glioma cells. The present study investigated how the expression of PDCD4 was silenced in glioma cells. It was hypothesized that HOTAIR may induce the recruitment of PRC2 to the PDCD4 promoter. Western blot analysis indicated that PDCD4 expression was upregulated when HOTAIR was knocked down in glioma cells (Fig. 5A). Histone modifications at the PDCD4 promoter were measured by ChIP assays. The results demonstrated that when HOTAIR was knocked down in glioma cells, the H3K27me3 level at the PDCD4 promoter was significantly reduced compared with the control ($P = 0.031$; Fig. 5B). Furthermore, the recruitment of EZH2 and SUZ12 to the PDCD4 promoter was measured, and the results indicated that the level of PRC2 components at the PDCD4 promoter was decreased in glioma cells following HOTAIR-knockdown compared with the control (Fig. 5C). These results suggest that the upregulation of HOTAIR results in PDCD4 silencing in glioma cells.

Discussion

Glioma is the most aggressive form of tumor located in the human brain, and despite advances in available therapies, glioma remains incurable (18). The tumors are particularly difficult to eradicate due to their highly invasive and metastatic

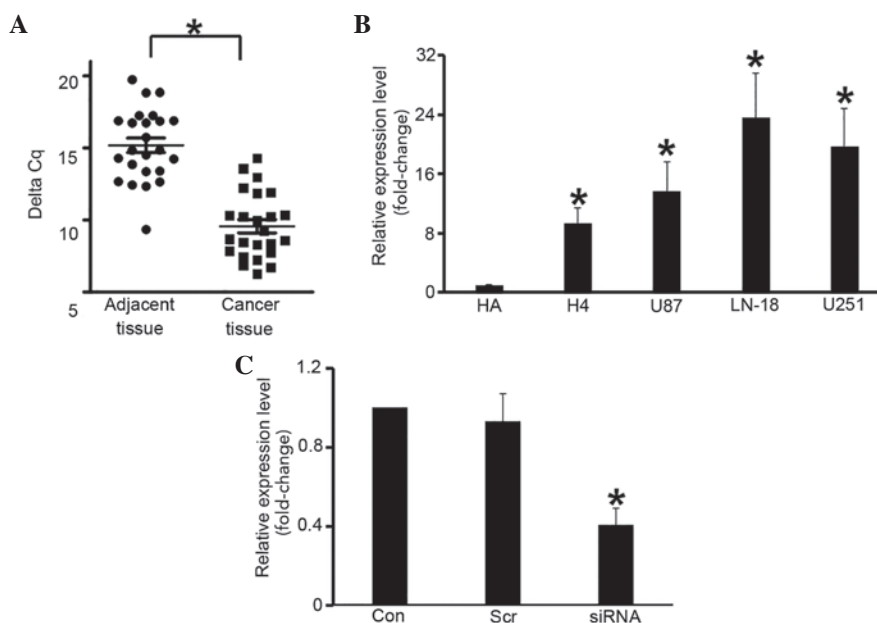


Figure 4. HOTAIR is upregulated in glioma cells. (A) Δ Cq values for HOTAIR RNA levels in 24 glioma tissue samples. (B) HOTAIR RNA levels in HA, H4, U87, LN-18 and U251 cells. Data represents the results of three independent experiments. (C) Relative expression levels of HOTAIR in U251 cells treated with siRNA targeting HOTAIR. * $P < 0.05$ vs. negative control. HOTAIR, homeobox transcript antisense RNA; Cq, quantification cycle; siRNA, small interfering RNA; Con, control; Scr, scramble.

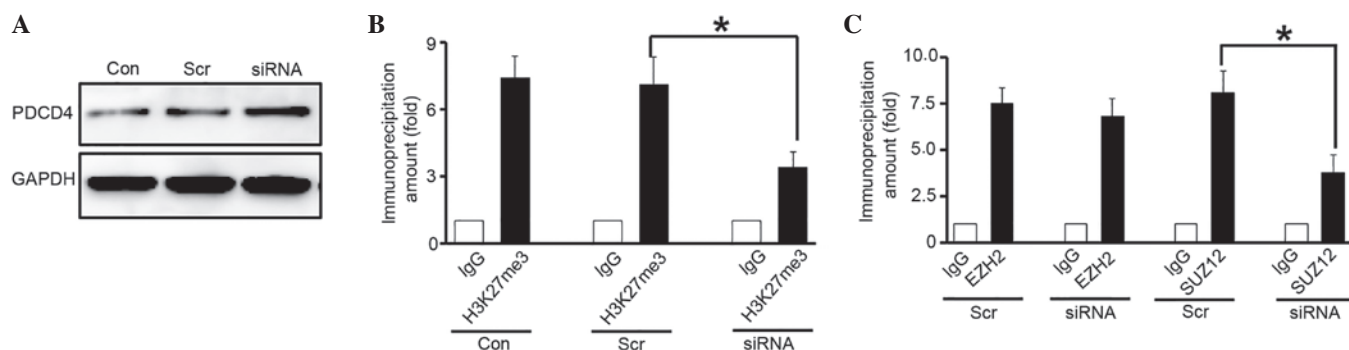


Figure 5. HOTAIR participates in the silencing of PDCD4 in glioma cells. (A) PDCD4 expression level in U251 cells treated with siRNA targeting HOTAIR. Scr RNA was used as the negative control. (B) ChIP analysis of H3K27me3 histone modification at the PDCD4 promoter in U251 cells treated with siRNA. (C) ChIP analysis of EZH2 and SUZ12 recruitment to the PDCD4 promoter in U251 cells treated with siRNA. All experiments were repeated three times. * $P < 0.05$ vs. negative control. HOTAIR, homeobox transcript antisense RNA; PDCD4, programmed cell death protein 4; siRNA, small interfering RNA; Scr, scramble; ChIP, chromatin immunoprecipitation; H3K27me3, histone 3 lysine 27 trimethylation; EZH2, enhancer of zeste homolog 2; SUZ12, suppressor of zeste 12; Con, control.

capabilities (19). In the present study, it was demonstrated that PDCD4 expression was suppressed in glioma cells, which suggested that PDCD4 may participate in glioma tumorigenesis.

As a potential tumor suppressor, PDCD4 regulates the expression of a variety of proteins, including p21 (20), urokinase receptor (21), hematopoietic progenitor kinase 1 (22), ornithine decarboxylase (23), carbonic anhydrase II (24) and c-Jun N-terminal kinase/c-Jun/activator protein 1 (25). In glioma cells, the function of PDCD4 remains poorly understood. Liwak *et al* (26) reported that the loss of PDCD4 expression contributes to increased chemotherapy resistance in glioblastoma multiforme by derepressing B-cell lymphoma-extra large translation. Gaur *et al* (27) reported that PDCD4 downregulation by miRNA-21 promotes glioblastoma proliferation *in vivo*. In the current

study, when PDCD4 was overexpressed in glioma cells, the proliferation rate and invasive capability significantly increased compared with the control. However, no information regarding the regulation of PDCD4 in glioma has been reported. The present study therefore proposes that PDCD4 regulation depends on alterations in histone modification at promoter region.

LncRNAs are generally defined as mRNA-like, non-protein coding transcripts that are >200 nucleotides in length (28,29). Using the most advanced sequencing platforms and algorithms for assembling transcripts from deep RNA-sequencing reads, it is estimated that there are ~20,000 distinct lncRNAs in humans (30). LncRNAs demonstrate unique profiles in different forms of human cancer, which serve as predictors of patient outcomes and reflect disease progression (31,32). It was recently identified

that lncRNAs function in a number of aspects of cell biology and may aid the development of tumors (33). HOTAIR, a well-studied lncRNA, has emerged as an important regulator of carcinogenesis and metastasis, and as a potential prognostic marker (34,35). Therefore, an increasing amount of research has focused on determining its functions, in addition to identifying its target genes. The present study observed that the expression of HOTAIR was dramatically upregulated in glioma cells compared with normal human astrocyte cells.

Regarding the function of HOTAIR, previous studies identified a possible role for HOTAIR in cancer. HOTAIR interacts with PRC2, which increases the level of H3K27me3, and subsequently decreases the expression of various genes, particularly metastasis-suppressing genes (36,37). Furthermore, the present study investigated the association between HOTAIR and PDCD4. The results demonstrated that the elevated expression of HOTAIR participated in PDCD4 regulation in a PRC2-dependent manner.

In conclusion, to the best of our knowledge, the current study investigated the association between the tumor suppressor PDCD4 and the lncRNA HOTAIR in glioma cells for the first time, with the results demonstrating that suppression of PDCD4 mediated by HOTAIR inhibits glioma cell proliferation and invasion in a PRC2-dependent manner. The results of the present study may aid the understanding of the detailed molecular mechanisms underlying glioma tumorigenesis, and support the notion that understanding the regulation of PDCD4 expression via HOTAIR intervention may contribute to the development of therapeutic strategies for the treatment of gliomas.

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