Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 272 (2008) 197–205
CANCER Letters
www.elsevier.com/locate/canlet

MicroRNA-21 down-regulates the expression of tumor suppressor PDCD4 in human glioblastoma cell T98G

Yang Chen [a,b,c], Wei Liu [a], Tengfei Chao [a,c], Yu Zhang [a,c], Xingqi Yan [a,c], Yanhua Gong [a], Boqin Qiang [a], Jiangang Yuan [a], Maosheng Sun [b,*], Xiaozhong Peng [a,*]

[a] *National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 5 Dong Dan San Tiao, Beijing 100005, PR China*
[b] *Department of Biochemistry and Molecular Biology, Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Kunming 650118, PR China*
[c] *Graduate School, Peking Union Medical College, Tsinghua University, Beijing 100005, PR China*

Received 30 April 2008; received in revised form 30 April 2008; accepted 30 June 2008

Abstract

MicroRNAs have been linked to different cancer-related processes. The microRNA miR-21 appears to function as an anti-apoptosis factor in glioblastomas. However, the functional target genes of miR-21 are largely unknown in glioblastomas. In this study, bioinformatics analysis was used to identify miR-21 target sites in various genes. Luciferase activity assay showed that a number of genes involved in apoptosis, PDCD4, MTAP, and SOX5, carry putative miR-21 binding sites. Expression of PDCD4 protein correlates inversely with expression of miR-21 in a number of human glioblastoma cell lines such as T98G, A172, U87, and U251. Inhibition of miR-21 increases endogenous levels of PDCD4 in cell line T98G and over-expression miR-21 inhibits PDCD4-dependent apoptosis. Together, these results indicate that miR-21 expression plays a key role in regulating cellular processes in glioblastomas and may serve as a target for effective therapies.

Keywords: Glioblastomas; miR-21; PDCD4; T98G

1. Introduction

Glioblastomas are the most common type of malignant primary brain tumors. Despite the advances in surgery, radiation therapy, and chemotherapy, the prognosis of patients with glioblastomas has not improved significantly over the past 20 years [1]. Understanding how the signaling pathways involved in migration, invasion, and apoptosis of glioblastomas are regulated is important for the development of more effective tumor therapies.

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs that regulate the stability or translational efficiency of target messenger RNAs[2]. A number of miRNAs have been

Abbreviations: miR-21, Hsa-miR-21; PDCD4, Programmed cell death 4.

* Corresponding authors. Tel.: +86 10 65296411; fax: +86 10 65240529 (X. Peng), tel.: +86 871 8334326; fax: +86 871 8334483 (M. Sun).
E-mail addresses: maoshs@imbcams.com.cn (M. Sun), pengxiaozhong@pumc.edu.cn (X. Peng).

0304-3835/$ - see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2008.06.034

identified from different human tumors and appear to play crucial roles in proliferation, differentiation, and apoptosis. One of these microRNAs, miR-21, is a key player in human cancers such as breast cancer, liver cancer, pancreatic cancer, colorectal cancer, and glioblastomas [3]. Chan et al. found that miR-21 is an anti-apoptosis factor in human glioblastoma cells. Knock-down of miR-21 in cultured glioblastoma cells triggers activation of caspases and leads to increased apoptotic cell death [4]. miR-21 knock-down disrupts glioma growth and displays synergistic cytotoxicity with S-TRAIL with in vivo xenografts of U87 cells [5]. Aberrantly expressed miR-21 down-regulates tumor suppressor PTEN and modulates gemcltabbine-induced apoptosis by PTEN-dependent activation of phosphoinositide 3-kinase (PI3K) signaling in cholangiocarcinoma and hepatocellular cancer [6,7]. Post-transcriptional down-regulation of tumor suppressor PDCD4 by miR-21 stimulates invasion, intravasation, and metastasis in colorectal cancer [8]. Expression of tumor suppressor Tropomyosin 1 (TPM1) and PDCD4 in breast cancer cell MCF-7 are down-regulated by expression of miR-21 [9,10] and knock-down of miR-21 expression suppresses both MCF-7 cells growth in vitro and tumor growth in the xenograft mouse model [11].

In this study, bioinformatics analysis was used to screen and identify various genes with miR-21 target sites. Luciferase activity assays indicated that PDCD4, MTAP, and SOX5 carry putative miR-21 binding sites. Furthermore, PDCD4 protein levels correlate inversely with miR-21 levels in human glioblastoma cell lines A172, T98G, U87, and U251. Reducing miR-21 increases PDCD4 in T98G cell line and over-expression of miR-21 inhibits PDCD4-dependent apoptosis.

2. Materials and methods

2.1. Cell lines

Human glioblastoma established cell lines A172, T98G, and U87 were purchased from American Type Culture Collection (Manassas, VA) and U251 was purchased from Cell center of Peking Union Medical College. Cell line 293ET was obtained from Dr. Chengyu Jiang (Peking Union Medical College). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. A172, T98G, U87, and U251 cells were maintained in Dulbecco's modified Eagle's Medium, and 293ET in Iscove's modified Dulbecco's medium. The media were supplemented with 10% fetal bovine serum (FBS), 5 mM L-glutamine, and 100 U/ml penicillin and 100 mg/ml streptomycin.

2.2. Plasmids and constructs

To construct a plasmid expressing miR-21, the genomic fragment containing miR-21 precursor (~400 bp) was amplified from T98G and cloned into pcDNA3.1(+) vector (Invitrogen). The expression of miRNA was detected by real-time PCR analysis, 48 h after transfection. The PCR primers are as follows: miR-21 FW, CGGGATCCTGGGG TTCGATCTTAACAGGC, miR-21 RV, CGGAA TTCCCACAATGCAGCTTAGTTTTCC.

Luciferase activity assay-related constructs were made by ligating 3′UTR fragments (about 300–1000 bp) containing the predicted binding sites of appropriate wild-type or relevant mutant forms downstream of the firefly luciferase reporter vector (pcDNA3.1-Luc) as previously described [12]. The sequences of these PCR primers are described in Supplementary Table 1.

To confirm whether the 3′UTR of PDCD4 contains a functional binding site for miR-21 in T98G cells, PDCD4 3′UTR or relevant sequences with mutations in the predicted miRNA binding site were cloned downstream of the GFP reporter of pcDNA3.1-GFP and transfected into T98G cells that express high levels of endogenous miR-21.

HA-tagged PDCD4 expression vector pcDNA3-HA-PDCD4 and empty vector pcDNA3 were kindly provided by Dr. Pagano (School of Medicine, New York University) [13]. To investigate the function of over-expressed miR-21 in PDCD4 induced apoptosis of T98G, wild-type PDCD4 3′UTR (~600 bp) and relevant mutant forms with mutation in the predicted miRNA binding site were cloned into the vector pcDNA3-HA-PDCD4 to create pcDNA3-HA-PDCD4-3UTR(WT) and pcDNA3-HA-PDCD4-3UTR(MT), respectively.

All PCR products were verified by DNA sequencing before cloning into the final destination vectors.

2.3. Luciferase activity assay

293ET cells were plated in 24-well plates, 1×10^5 in each well. After 24 h, cells were co-transfected with 1 μg of firefly luciferase reporter vector containing 3′UTR of candidate genes, 0.2 μg of the transfection

control vector pTK containing *Renilla* luciferase and 1 μg pcDNA3.1-miR-21 or vehicle control pcDNA 3.1. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega) 48 h after transfection. The firefly luciferase activity was normalized to the *Renilla* luciferase activity as an internal transfection control. Then, the luciferase values were normalized to the average values for the corresponding vehicle control transfections. Values represent mean ± SD of three experiments from three independent transfections.

2.4. Northern blot

Total RNA was extracted from cells with Trizol Reagent (Invitrogen) according to the manufacturer's instruction and 20 μg of total RNA was resolved on 15% polyacrylamide urea gels and transferred to Hy-Bond N^+ nylon membrane. Oligonucleotide complementary to the mature miR-21 was 5′ end labeled with [γ-^{32}P]adenosine triphosphate (ATP) by T4 kinase (TaKaRa). The membranes were incubated with labeled probe (1.5×10^6 CPM/ml hybridization buffer) prior to visualization using phosphorimaging. Before transferring to membrane, gels were stained with ethidium bromide and 5S RNA was used as loading control. Probes are Anti-miR-21, 5′-TCAACAT CAGTCTGATAAGCTA-3′.

2.5. Western blot

Proteins from cells and tissue samples were extracted with RIPA lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 1% NP40, 1% deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche)). Proteins from total cell lysates were resolved by 10% SDS–PAGE gel, transferred to the nitrocellulose membrane, blocked in 5% non-fat milk in PBS/Tween-20, and blotted with the antibody against PDCD4 (1:2000, Abcam), and blotted with Goat anti Rabbit IgG (1:3000, Santa Cruz). Visualization of the second antibody was performed using a chemiluminescence detection procedure according to the manufacturer's protocol (Amersham Pharmacia Biotech). β-Actin was used as loading control.

2.6. Semi-quantitative RT-PCR

Total RNA was extracted from cells or tissue samples with Trizol Reagent (Invitrogen), and 2 μg samples were reverse-transcribed to cDNA by using reverse transcriptase (NEB, USA). In each analysis, 2.5% of the cDNA pool was used for amplification and PCR was run for 25–30 cycles. The semi-quantitative RT-PCR primers are as follows: PDCD4 FW, 5′-TGGATTAACTGTGCCAACCA-3′; RV, 5′-CCACCAACTGTGGTGCTCTA-3′; GAPDH FW, 5′-ACCACAGTCCATGCCATCAC-3′, RV, 5′-TCCACCACCCTGTTGCTGTA-3′.

2.7. miRNA real-time PCR

Stem-loop RT-PCR for mature miR-21 was performed as previously described [14]. PCR was performed using SYBR Premix Ex Taq™ (Takara) according to the manufacture. RNA input was normalized by human U6 snRNA. The sequences of the primers are described in Supplementary Table 1.

2.8. Oligonucleotide transfection

Oligonucleotides 2′-*O*-methyl(2′-*O*-Me)-Anti-miR-21, 5′-UCAUACAGCUAGAUAACCAAAG A-3′ and negative control 2′-*O*-Me-NC, 5′-UUCU CCGAACGUGUCACGUTT-3′ were chemically synthesized by Shanghai Genechem Co., Ltd. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) and medium was replaced 8 h later and cells were collected for the next experiments 48 h post-transfection. The final concentrations of 2′-*O*-Me-Anti-miR-21 and negative control 2′-*O*-Me-NC were 50 nmol/L except when noted.

2.9. TUNEL

T98G cells were plated on glass coverslips in 12-well plates, 2×10^5 in each well. After 24 h, cells were transfected with 1 μg of pcDNA3.1 or pcDNA3.1-miR-21 and 1.5 μg of pcDNA3-HA-PDCD4-3UTR (WT) or pcDNA3-HA-PDCD4-3UTR(MT). The cells on the microcover glasses were fixed with 4% paraformaldehyde for 24 h and permeabilized with 0.1% Triton X-100 in PBS, then washed with PBS. Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining was performed 48 h post-transfection using the In Situ Cell Death Detection Kit, TMR Red (Roche) according to the manufacturer's protocol. The number of TUNEL-positive cells were divided by the number of 4′,6-diamidino-2-phenylindole-

stained cells to yield the percent apoptotic nuclei. Three 20× objective fields containing ~600 cells each were counted per coverslip, with three coverslips analyzed per condition.

2.10. Databases and GenBank accession number

The miR-21 sequence was obtained from miRBase (http://www.microrna.sanger.ac.uk). The target genes of miR-21 were predicted with miRanda (http://www.cbio.mskcc.org/mirnaviewer), PicTar (http://www.pictar.bio.nyu.edu), TargetScan (http: //www.targetscan.org).

2.11. Statistics

Results were expressed as means ± SD unless indicated otherwise. Differences between groups were assessed by unpaired, two-tailed Student's *t*-test, $p < 0.05$ was considered significant.

3. Results

3.1. Identification of miR-21 possible target genes

It is established that miR-21 is aberrantly expressed in various types of cancers [3,15]. Inhibition of miR-21 leads to increased apoptosis in glioblastomas [4] and attenuated intravasation and metastatic capacity in colon cancer [8]. To examine the potential involvement of miR-21 and its targets in glioblastomas, MiRanda, TargetScan, and Pictar were used to analyze the possible target genes of miR-21 (Fig. 1A). Our analysis showed that there were 119 candidates in the intersection of at least two programs and 26 candidates in the intersection of three programs (Supplementary Table 2). Results were identified with Online Mendelian Inheritance in Man™ (OMIM™) and eight possible candidates related to glioblastomas or apoptosis were selected.

Before the luciferase activity assay, we constructed a plasmid expressing miR-21, the genomic fragment of miR-21 precursor (~400 bp) from T98G was amplified. The ectopic expression of miR-21 in 293ET was confirmed

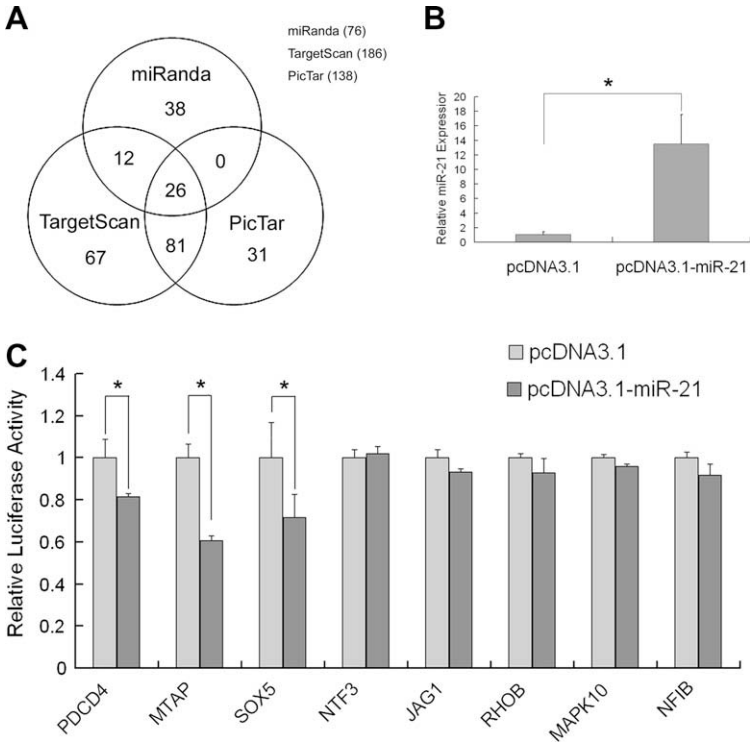

Fig. 1. Identification of miR-21 possible target genes. (A) Diagram showing the predicted results from databases miRanda, TargetScan, and PicTar. (B) Ectopic expression of miR-21. The genomic fragment containing miR-21 precursor (~400 bp) was cloned into pcDNA3.1(+) vector and miRNA expression was detected by Real-time PCR analysis, 48 h after transfection. (C) Luciferase activity reporter assays. Constructs of 3′UTR sequences from the indicated genes were co-transfected into 293ET cells along with a *Renilla* luciferase transfection control plasmid together with pcDNA3.1 or pcDNA3.1-miR-21. The values were normalized to the average values for the corresponding empty vector transfections. Data are shown as mean ± SD of three replicates and are representative of three independent experiments ($^{*}P < 0.05$).

by real-time PCR (Fig. 1B). We then tested eight candidate genes by cloning their 3′UTRs fragments into downstream of pcDNA3.1-Luc and co-transfected with pcDNA3.1 or pcDNA3.1-miR-21 into 293ET cells. The luciferase values were normalized to the average values for the corresponding empty vector pcDNA3.1 transfected into 293ET cells. Luciferase activity with 3′UTRs of PDCD4, MTAP and SOX5 were reduced by a transient transfection with pcDNA3.1-miR-21, but there were no changes detected in the remaining five candidates (Fig. 1C). Thus PDCD4, MTAP, and SOX5 3′UTRs carry miR-21 regulatory elements.

3.2. PDCD4, MTAP, and SOX5 carry putative miR-21 binding sites

To confirm there are miR-21 binding sites in the 3′UTRs of PDCD4, MTAP, and SOX5, we further identified the binding site with the miRanda program and compared the nucleotide sequences in different species. The sequences for the binding sites in the 3′UTRs of PDCD4, MTAP, and SOX5 are highly conserved among different specie (Fig. 2A). We cloned these three 3′UTRs and their relevant mutants into luciferase report vector pcDNA3.1-Luc. Luciferase activity assay showed that ectopically expressed miR-21 decreased the luciferase activity of 3′UTRs with wild-type binding sites, but ectopically expressed miR-21 had no effect on 3′UTRs with mutant type binding sites (Fig. 2B). These results suggested that PDCD4, MTAP, and SOX5 carry putative mir-21 binding sites and can be regulated by miR-21.

3.3. miR-21 down-regulates PDCD4 at translational level in T98G

Many (14/30 or 47%) of human glioma samples show a significant decline in PDCD4 mRNA, but more samples (23/30 or 77%) display little PDCD4 protein expression [16]. To investigate the relationship between miR-21 and PDCD4 in glioblastomas, we analyzed the expression of miR-21 and PDCD4 in glioblastoma cell lines A172, T98G, U87, and U251 and nonneoplastic control 293ET. miR-21 levels were markedly elevated in four established glioblastoma cell lines A172, T98G, U87,

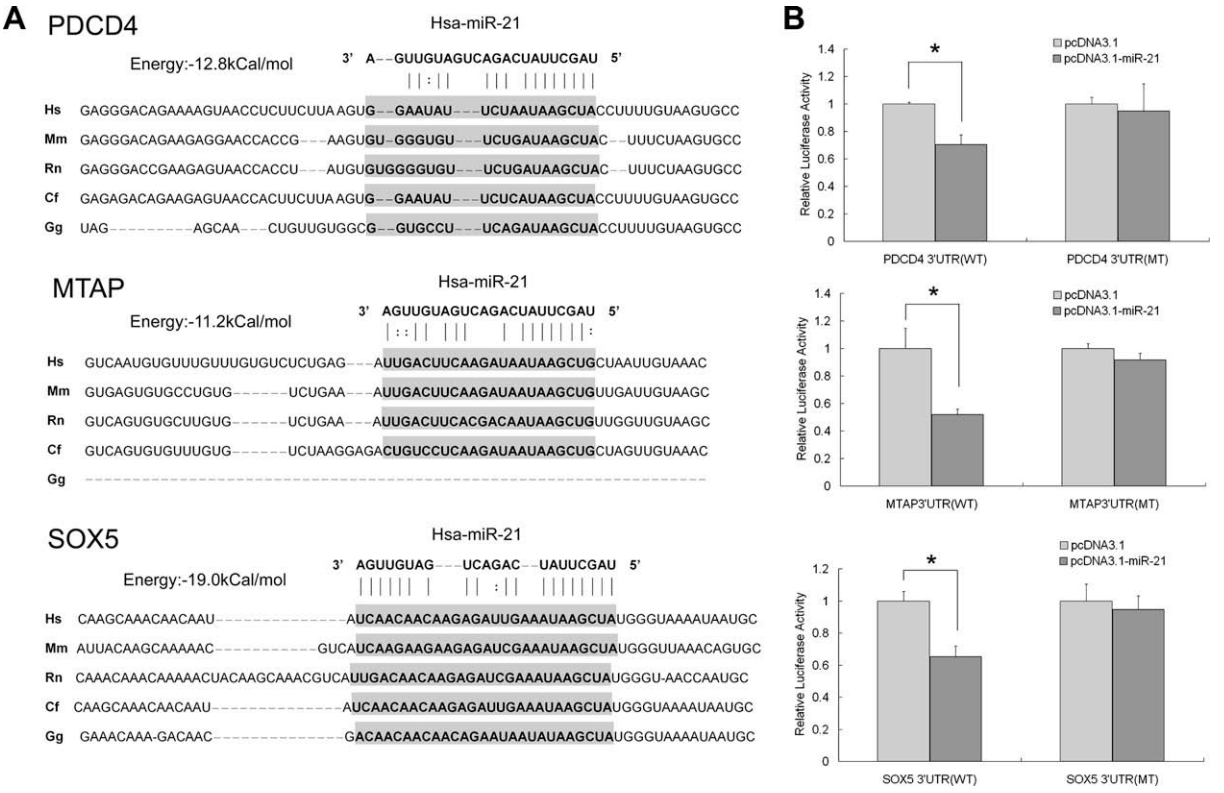

Fig. 2. (A) miR-21 binding sites in PDCD4, MTAP, and SOX5 3′UTR were identified with miRanda database. The nucleotide sequence for miR-21 and its target in five species (Hs, *Homo sapiens*; Mm, *Mus musculus*; Rn, *Rattus norvegicus;* Cf, *Canis familiaris*; Gg, *Gallus gallus*) were compared and PDCD4, MTAP, and SOX5 3′UTR and their relevant mutants were cloned into pcDNA3.1-Luc. (B) Luciferase activities of PDCD4, MTAP, and SOX5 3′UTR (wild-type) were regulated by over-expression of miR-21 ($^{*}P < 0.05$), but were not affected for 3′UTRs with mutated forms of the binding sites (gray sequences are deleted).

and U251 compared with cultured nonneoplastic cell line 293ET (Fig. 3A). PDCD4 protein was absent in the glioblastoma cell lines and present in cell line 293ET (Fig. 3B). Semi-Quantitative-RT-PCR showed that there were no significant differences at PDCD4 mRNA levels between these cell lines (Fig. 3C). These initial experiments suggested that PDCD4 protein levels correlate inversely with miR-21 levels in glioblastoma cell lines.

To explore whether PDCD4 protein expression is down-regulated by miR-21 in glioblastoma cells, miR-21 was knocked down with 2′-*O*-Me-Anti-miR-21 in T98G cells. The protein level of PDCD4 was restored in response to miR-21 knock-down, but there was no change in the PDCD4 mRNA level (Fig. 3D). Reducing miR-21 resulted in a corresponding increase in endogenous protein levels, relative to the effect of the scrambled control.

To insure that 3′UTR of PDCD4 contains a functional binding site for miR-21, we cloned PDCD4 3′UTR and its relevant mutant with mutation in the predicted miRNA binding site and inserted them downstream of the GFP reporter of the pcDNA3.1-GFP construct and transfected them into T98G cells which express high levels of endogenous miR-21. We also co-transfected GFP reporter vector containing wild-type PDCD4 3′UTR and two concentrations (20 or 50 nM) of 2′-*O*-Me-miR-21 into T98G cells. High levels endogenous miR-21 inhibited the expression of GFP with wild-type PDCD4 3′UTR and no effect on GFP expression was detected with mutant forms of PDCD4 3′UTR. On the other hand, miR-21 knock-down increased the expression of GFP with wild-type PDCD4 3′UTR (Fig. 3E). The putative miR-21 binding site within PDCD4 3′UTR was responsible for miR-21-mediated translational repression of GFP in T98G. Thus, miR-21 down-regulates PDCD4 at the translational level in T98G glioblastoma cell line.

3.4. Over-expressed miR-21 inhibits PDCD4-dependent apoptosis in T98G

The tumor suppressor PDCD4, which is down-regulated in breast cancer, colorectal cancer and glioma, inhibits transformation and invasion, and induces apoptosis in different tumors [17]. We employed T98G cells, and transfected cells with pcDNA3-HA-PDCD4 (Fig. 4A) and cell apoptosis were analyzed with TUNEL. We found that over-expressed PDCD4 increased the apoptosis of T98G cells (Fig. 4B), suggesting that PDCD4 is an apoptosis factor in T98G cells.

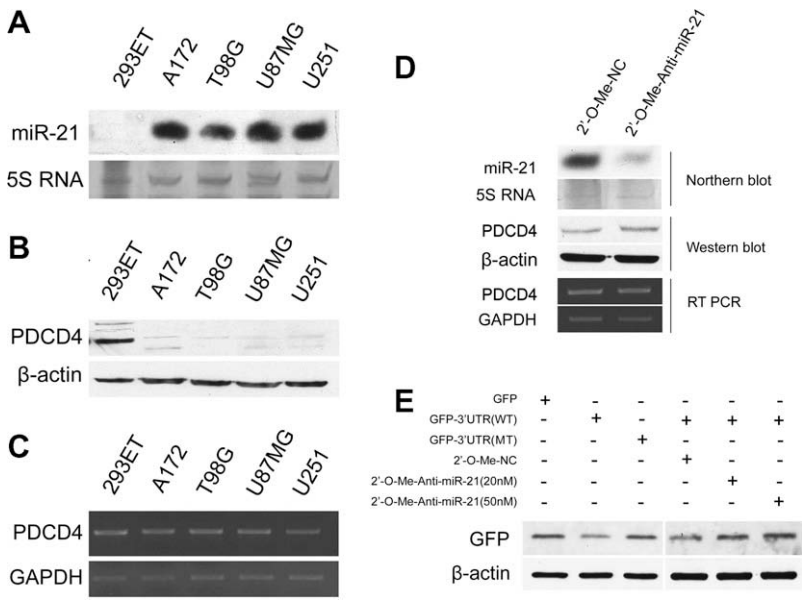

Fig. 3. PDCD4 protein level correlated inversely with miR-21 level in glioblastoma cell lines and was down-regulated by miR-21 at the translational level. (A) Northern blot analysis of miR-21 transcription in nonneoplastic control 293ET and glioblastoma cell lines A172, T98G, U87, and U251. 5S RNA transcription was used as loading control. (B and C) Western blot and semi-quantitative RT-PCR show the expression of PDCD4 at protein and mRNA levels. (D) Northern blot of miR-21 expression in T98G cells treated with 2′-*O*-Me-NC and 2′-*O*-Me-Anti-miR-21. 5S RNAs were used as loading control. miR-21 knock-down increased the expression of PDCD4 protein levels in T98G cells and there was no change in the level of PDCD4 mRNA. (E) Putative miR-21 binding site within PDCD4 3′UTR was responsible for repression of GFP expression in T98G cells. PDCD4 3′UTR (~600 bp) and its relevant mutant were cloned and inserted downstream of the GFP reporter of pcDNA3.1-GFP and transfected into T98G cells, which possess high level of endogenous miR-21. Meanwhile, GFP reporter vector containing wild-type PDCD4 3′UTR and 2′-*O*-Me-miR-21(20 or 50 nM) were co-transfected into T98G cells.

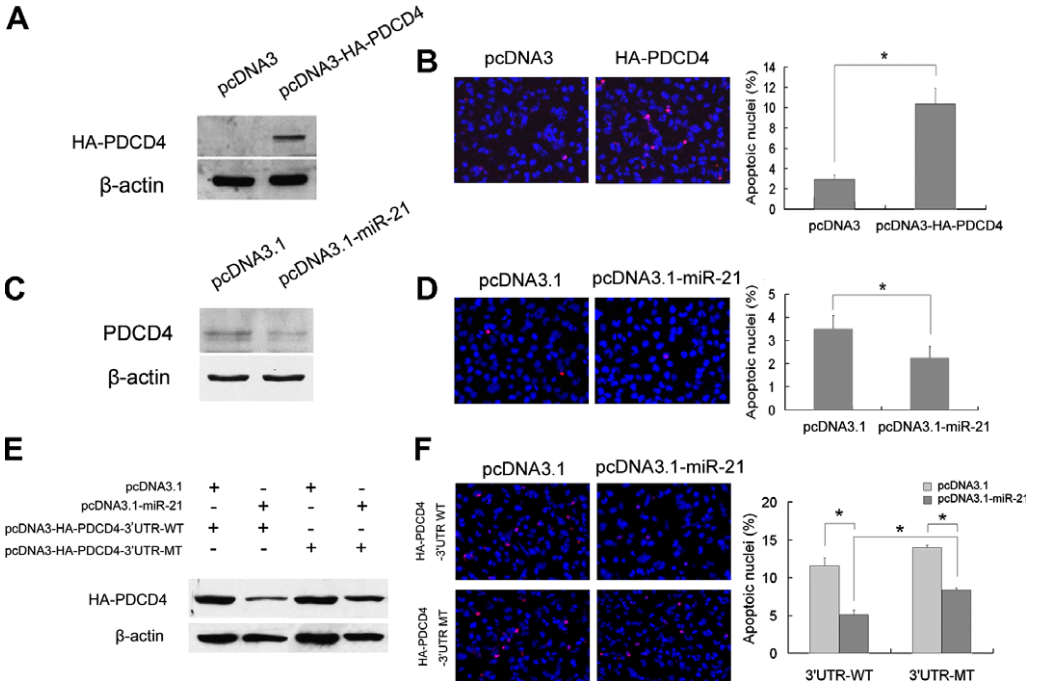

Fig. 4. Over-expression of miR-21 inhibited PDCD4-dependent apoptosis of T98G cells. (A) Ectopic expression of HA-PDCD4 in T98G was determined by Western blot. (B) Ectopic expression of HA-PDCD4 increased apoptosis of T98G cells. Representative images of apoptotic nuclear TUNEL TMR (Red) staining was visualized and cells were counterstained with 4′,6-diamidino-2-phenylindole(DAPI) to highlight nuclei. Three 20× objective fields containing ~600 cells each were counted per coverslip, with three coverslips analyzed per condition. ($^{*}P < 0.05$). (C and D) Over-expression of miR-21 inhibited the endogenous expression of PDCD4, and decreased T98G cell apoptosis ($^{*}P < 0.05$). (E) Expression of HA-PDCD4 with wild-type 3′UTR was down-regulated and expression with the mutant form of the miR-21 binding site abolished the effect of miR-21 on HA-PDCD4 expression. (F) Over-expression of miR-21 inhibited the expression of PDCD4 with wild-type 3′UTR and decreased apoptosis ($^{*}P < 0.05$). Over-expression of miR-21 did not affect the expression of PDCD4 with mutant 3′UTR but decreased apoptosis ($^{*}P < 0.05$). Over-expression of miR-21 significantly affected apoptosis for cells expressing PDCD4 with wild-type 3′UTR compared to cell expressing PDCD4 with mutant 3′UTR 3′UTR ($^{*}P < 0.05$). (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Previous studies have indicated that miR-21 is an anti-apoptosis factor in glioblastoma cells, and miR-21 knock-down increases apoptosis [4]. In our study, over-expression of miR-21 inhibits the endogenous expression of PDCD4 (Fig. 4C), and decreases apoptosis (Fig. 4D). Thus high level of miR-21 maintains lower level of apoptosis in T98G cells.

To investigate the biological function played by miR-21 and its target PDCD4 in T98G cells, pcDNA3-HA-PDCD4-3′UTR(WT) or pcDNA3-HA-PDCD4-3′UTR (MT) were co-transfected with negative control vector pcDNA3.1 or ectopic-expression vector pcDNA3.1-miR-21. Western blot reveals that expression of HA-PDCD4 from mRNA containing the wild-type 3′UTR was down-regulated, while expression of HA-PDCD4 from mRNA containing a mutation in the miR-21 binding was no longer affected by miR-21 expression (Fig. 4E). The TUNEL assay indicated that over-expression of miR-21 that inhibits the expression of PDCD4 with wild-type 3′UTR resulted in a decrease in apoptosis. The over-expression of miR-21, which did not affect the expression of PDCD4 with mutant 3′UTR continued to have an impact on apoptosis (Fig. 4F). These results indicated that PDCD4 is a direct functional target of miR-21 in T98G cells and that over-expression of miR-21 not only down-regulates PDCD4 but also may regulate the expression of other proteins that affect apoptosis.

4. Discussion

A better understanding of the molecular mechanisms occurring in glioblastomas has the potential of helping the development of new and various targeted molecular therapies. In this study, we identified functional targets of an anti-apoptosis factor miR-21. Although more than several hundred candidate target genes which might carry miR-21 binding sites based on the combined analysis of miRanda, PicTar, and TargetScan programs, not every putative site is a functional miRNA target.

Our results indicate that a subset of genes with putative binding sites for miR-21 is likely to participate in the regulation of apoptosis.

PDCD4 was first isolated from a mouse cDNA library as a tumor-related gene [18]. PDCD4 is known as a pro-apoptotic molecule and is involved in inhibiting neoplastic transformation or decrease anchorage in dependent colonies [17]. PDCD4 is down-regulated post-transcriptionally by miR-21 and induces invasion-related processes in colorectal cancer [8]. miR-21 also down-regulates PDCD4 protein levels in breast cancer [19]. Our results indicate that PDCD4 protein levels not only correlate inversely with miR-21 levels in a number of tumor cell lines, but PDCD4 mRNA is a direct functional target of miR-21 in glioblastoma cell line T98G.

How does miR-21expression result in an anti-apoptosis signal? It is very likely that it regulates apoptosis by interfering with the expression of a number of proteins, some of which are tissue specific, that are linked to apoptosis. Meng et al. found that high level of miR-21 inhibits the expression of PTEN and activates the PI3K signaling in cholangiocarcinoma cell lines. Si et al. found that anti-miR-21 suppressed both cell growth in vitro and tumor growth in the breast cancer xenograft mouse model, which may be mediated by a down-regulation of the anti-apoptotic protein Bcl-2. In our study, we also found that MTAP and SOX5 carry putative mir-21 binding sites and are regulated by miR-21 in the luciferase activity assay. Besides PDCD4, MTAP may also function as an anti-apoptotic factor. MTAP plays a major role in polyamine metabolism [20]. MTAP catalyses the phosphorylation of MTA, a by-product of the synthesis of polyamines and is a methyl-transferase inhibitor. Many malignant cells lack MTAP activity [21] and accumulate MTA. We propose that miR-21 might inhibit the expression of MTAP thereby increasing the accumulation of MTA. Accumulation of MTA would then interfere with IFN-induced JAK/STAT signaling and decrease apoptosis because of its ability to inhibit methyl-transferases. Mowen et al. found that arginine methylation of STAT1 by the protein arginine methyl-transferase PRMT1 is required for IFN-induced JAK/STAT signaling [22]. SOX5, a member of the group D Sox factors, participates in BMP signaling and is responsible for mesodermal limb chondrogenesis [23]. Stolt et al. found that Sox5 jointly regulates several stages of oligodendrocyte development in mouse spinal cord [24]. We propose that miR-21 might inhibit the expression SOX5 and affect its downstream signaling which interferes with normal cell processes.

In the xenograft carcinoma model, one transient transfection with anti-miR-21 is sufficient to cause substantial inhibition of breast tumor growth [11]. Moreover, it was shown that 5-fluorouracil can up-regulate miR-21 which is already over-expressed in neoplastic tissues [25]. It is possible that drug-induced miRNA gene hyper expression could induce toxic responses. In contrast, a drug might influence the expression of miRNA genes in a direction that is opposite to that induced by neoplastic transformation. A recent study showed that altered levels of let-7i, miR-16, and miR-21 affected the potencies of a number of anticancer agents by up to fourfold. This raises the possibility that anti-miR-21 may have potential therapeutic value as many microRNAs [5]. In vivo studies using experimental glioblastoma and systemic or local application of an miR-21-antagonist will be needed.

In summary, we report that PDCD4, MTAP, and SOX5 carry putative miR-21 binding sites. PDCD4 is a direct functional target of miR-21 in T98G, and over-expression of miR-21 may activate multiple pathways to act as an anti-apoptosis factor. Together, these results suggest that altering the processes controlled by miR-21 by regulating its expression or interaction with downstream targets would be a potential chemotherapeutic agent for fighting glioblastomas.

Conflict of interest

None declared.

Acknowledgements

This work was supported by funds from National Natural Sciences Foundation of China (Nos. 30608022, 90612019, 3043000, 30721063), "863" project (Nos. 2006AA0Z137, 006AA02A304), "973" project (Nos. 2004CB518604, 2005CB2507, 2006CB504100, 2007CB946902, 2007CB946900), Program for New Century Excellent Talents in University (Nos. NCET-07-0505). We thank Prof. Samuel Schacher, Department of Neuroscience, Columbia University for proofreading the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2008.06.034.

References

[1] D.N. Louis, Molecular pathology of malignant gliomas, Annu. Rev. Pathol. 1 (2006) 97–117.

[2] A.B. Shyu, M.F. Wilkinson, A. van Hoof, Messenger rna regulation: to translate or to degrade, EMBO J. 27 (2008) 471–481.

[3] W.C. Cho, Oncomirs: the discovery and progress of micrornas in cancers, Mol. Cancer 6 (2007) 60.

[4] J.A. Chan, A.M. Krichevsky, K.S. Kosik, Microrna-21 is an antiapoptotic factor in human glioblastoma cells, Cancer Res. 65 (2005) 6029–6033.

[5] M.F. Corsten, R. Miranda, R. Kasmieh, A.M. Krichevsky, R. Weissleder, K. Shah, Microrna-21 knockdown disrupts glioma growth in vivo and displays synergistic cytotoxicity with neural precursor cell delivered s-trail in human gliomas, Cancer Res. 67 (2007) 8994–9000.

[6] F. Meng, R. Henson, M. Lang, H. Wehbe, S. Maheshwari, J.T. Mendell, et al., Involvement of human micro-rna in growth and response to chemotherapy in human cholangiocarcinoma cell lines, Gastroenterology 130 (2006) 2113–2129.

[7] F. Meng, R. Henson, H. Wehbe-Janek, K. Ghoshal, S.T. Jacob, T. Patel, Microrna-21 regulates expression of the pten tumor suppressor gene in human hepatocellular cancer, Gastroenterology 133 (2007) 647–658.

[8] I.A. Asangani, S.A. Rasheed, D.A. Nikolova, J.H. Leupold, N.H. Colburn, S. Post, et al., Microrna-21 (mir-21) post-transcriptionally downregulates tumor suppressor pdcd4 and stimulates invasion intravasation and metastasis in colorectal cancer, Oncogene 27 (2008) 2128–2136.

[9] S. Zhu, M.L. Si, H. Wu, Y.Y. Mo, Microrna-21 targets the tumor suppressor gene tropomyosin 1 (tpm1), J. Biol. Chem. 282 (2007) 14328–14336.

[10] Z. Lu, M. Liu, V. Stribinskis, C.M. Klinge, K.S. Ramos, N.H. Colburn, et al., Microrna-21 promotes cell transformation by targeting the programmed cell death 4 gene, Oncogene 27 (2008) 4373–4379.

[11] M.L. Si, S. Zhu, H. Wu, Z. Lu, F. Wu, Y.Y. Mo, Mir-21-mediated tumor growth, Oncogene 26 (2007) 2799–2803.

[12] X. Yan, T. Chao, K. Tu, Y. Zhang, L. Xie, Y. Gong, et al., Improving the prediction of human microrna target genes by using ensemble algorithm, FEBS Lett. 581 (2007) 1587–1593.

[13] N.V. Dorrello, A. Peschiaroli, D. Guardavaccaro, N.H. Colburn, N.E. Sherman, M. Pagano, S6k1- and betatrcp-mediated degradation of pdcd4 promotes protein translation and cell growth, Science 314 (2006) 467–471.

[14] C. Chen, D.A. Ridzon, A.J. Broomer, Z. Zhou, D.H. Lee, J.T. Nguyen, et al., Real-time quantification of microrna by stem-loop rt-pcr, Nucleic Acids Res. 33 (2005) e179.

[15] S. Fujita, T. Ito, T. Mizutani, S. Minoguchi, N. Yamamichi, K. Sakurai, et al., Mir-21 gene expression triggered by ap-1 is sustained through a double-negative feedback mechanism, J. Mol. Biol. 378 (2008) 492–504.

[16] F. Gao, P. Zhang, C. Zhou, J. Li, Q. Wang, F. Zhu, et al., Frequent loss of pdcd4 expression in human glioma: Possible role in the tumorigenesis of glioma, Oncol. Rep. 17 (2007) 123–128.

[17] B. Lankat-Buttgereit, R. Goke, Programmed cell death protein 4 (pdcd4): a novel target for antineoplastic therapy?, Biol Cell 95 (2003) 515–519.

[18] K. Shibahara, M. Asano, Y. Ishida, T. Aoki, T. Koike, T. Honjo, Isolation of a novel mouse gene ma-3 that is induced upon programmed cell death, Gene 166 (1995) 297–301.

[19] L.B. Frankel, N.R. Christoffersen, A. Jacobsen, M. Lindow, A. Krogh, A.H. Lund, Programmed cell death 4 (pdcd4) is an important functional target of the microrna mir-21 in breast cancer cells, J. Biol. Chem. 283 (2008) 1026–1033.

[20] H.G. Williams-Ashman, J. Seidenfeld, P. Galletti, Trends in the biochemical pharmacology of 5′-deoxy-5′-methylthioadenosine, Biochem. Pharmacol. 31 (1982) 277–288.

[21] T. Nobori, K. Takabayashi, P. Tran, L. Orvis, A. Batova, A.L. Yu, et al., Genomic cloning of methylthioadenosine phosphorylase: a purine metabolic enzyme deficient in multiple different cancers, Proc. Natl. Acad. Sci. USA 93 (1996) 6203–6208.

[22] K.A. Mowen, J. Tang, W. Zhu, B.T. Schurter, K. Shuai, H.R. Herschman, et al., Arginine methylation of stat1 modulates ifnalpha/beta-induced transcription, Cell 104 (2001) 731–741.

[23] J. Chimal-Monroy, J. Rodriguez-Leon, J.A. Montero, Y. Ganan, D. Macias, R. Merino, et al., Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: sox genes and bmp signaling, Dev. Biol. 257 (2003) 292–301.

[24] C.C. Stolt, A. Schlierf, P. Lommes, S. Hillgartner, T. Werner, T. Kosian, et al., Soxd proteins influence multiple stages of oligodendrocyte development and modulate soxe protein function, Dev. Cell 11 (2006) 697–709.

[25] L. Rossi, E. Bonmassar, I. Faraoni, Modification of mir gene expression pattern in human colon cancer cells following exposure to 5-fluorouracil in vitro, Pharmacol. Res. 56 (2007) 248–253.