Oncology Research, Vol. 27, pp. 691–701
Printed in the USA.

0965-0407/19 $90.00 + .00
DOI: https://doi.org/10.3727/096504018X15426763753594
E-ISSN 1555-3906
www.cognizantcommunication.com

MicroRNA-101 Targets CXCL12-Mediated Akt and Snail Signaling Pathways to Inhibit Cellular Proliferation and Invasion in Papillary Thyroid Carcinoma

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Escalating evidence suggests that microRNA-101 (miR-101) is implicated in the development and progression of various cancers, including papillary thyroid carcinoma (PTC). However, the biological function and molecular mechanisms of miR-101 in PTC are still unclear. In this study, we demonstrated that miR-101 expression was significantly decreased in PTC tissues and cell lines. Clinically, a low level of miR-101 was positively associated with advanced histological stages and lymph node and distant metastases. The expression of CXCL12 was negatively correlated with miR-101 level in PTC. CXCL12 was validated as a direct target of miR-101 in PTC cells. Functional experiments proved that miR-101 markedly reduced the proliferation, apoptosis escape, migration, and invasion of PTC cells. Moreover, CXCL12 restoration rescued the suppressive effects of miR-101 on PTC cells by activating Akt- and EMT-associated signaling pathways. Overall, miR-101 exerts oncostatic effects on PTC by downregulating CXCL12 and repressing its downstream Akt and Snail signaling pathways, suggesting that miR-101/CXCL12/Akt or Snail axis may serve as a potential therapeutic target for PTC.

Key words: miR-101; Papillary thyroid carcinoma (PTC); CXCL12; Growth; Metastasis; Akt signaling

INTRODUCTION

Thyroid cancer (TC) is one of the most common endocrine malignancies with steadily increased incidence over the past few decades worldwide[1]. Papillary thyroid carcinoma (PTC) is the most prevalent type of TCs, accounting for ~80% of all TC cases[2]. The prognosis of most PTC patients is often favorable after surgical resection combined with radio- or chemotherapy. However, lymph node or distant metastases and recurrence often occur in the 10%–15% of PTC cases who have poor outcomes[3–5]. Therefore, it is necessary and vital to explore the mechanisms involved in PTC carcinogenesis and to identify new therapeutic targets.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate gene expression through interacting with the 3′-untranslated regions (3′-UTRs) of target mRNAs by inducing translational repression or mRNA degradation[6–8]. miRNAs are involved in diverse cellular processes, such as proliferation, apoptosis, invasion, and migration[9,10]. Recently, miRNAs may serve as oncogenes or tumor suppressors in many types of cancers by regulating their direct targets[10,11]. In the literature, miRNA-101 (miR-101) affords tumor-suppressive roles in a variety of human malignancies, such as breast cancer[12], colon cancer[13], lung cancer[14], hepatocellular carcinoma[15], gastric cancer[16], and PTC[17–20]. Reportedly, miR-101 inhibits PTC cell proliferation, migration, and invasion by targeting Rac1[17,18], hinders tumorigenesis of PTC in vitro and in vivo by downregulating USP22[19], and promotes TRAIL-induced mitochondrial apoptosis of PTC cells by targeting c-met and Mcl-1[20]. However, knowledge is limited on the mechanisms by which miR-101 reduces the vicious behaviors of PTC.

Chemokines are a family of small cytokines and were initially characterized by their ability to promote chemotaxis[21]. Chemokine (C-X-C motif) ligand 12 (CXCL12), also known as stromal cell-derived factor-1 (SDF-1), is a member of the CXC subfamily of chemokines that binds to its receptors CXC chemokine receptor 4 (CXCR4) or CXCR7 to drive cell migration[22,23]. Mounting evidence addresses that binding of CXCL12 to CXCR4 and/or CXCR7 mediates a broad range of cellular functions, including proliferation, survival, adhesion, and migration[23,24]. CXCL12 is highly expressed in multiple

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cancers, such as pancreatic cancer[25], glioblastoma[26], prostate cancer[27], breast cancer[28], and PTC[29–31]. High CXCL12 and CXCR7 levels were positively correlated with lymph node metastasis in PTC[29]. Also, CXCL12 may act as a diagnostic marker for PTC[30,31]. Lin et al.[32] reported that the CXCL12/CXCR4 axis accelerates migration, invasion, and epithelial–mesenchymal transition (EMT) in PTC cells. However, the biological roles of CXCL12 in PTC remain largely unknown.

In this study, we ascertained the decreased miR-101 expression, increased CXCL12 level, and an inverse correlation between the levels of miR-101 and CXCL12 in PTC cells and tissues. Downregulation of miR-101 was negatively associated with advanced histological stages and metastases. CXCL12 was confirmed as a direct target of miR-101 in PTC cells. In vitro, overexpression of CXCL12 counteracted miR-101-mediated inhibition of proliferation, migration, and invasion and enhancement of apoptosis in PTC cells. Molecularly, miR-101 led to notable reduction in levels of CXCL12, phosphorylated (p)-Akt (Ser473), cyclin D1, Bcl-2, vimentin, and Snail and increase in Bax and E-cadherin expression in PTC cells, which were reversed by CXCL12 restoration. Clinically, the levels of CXCL12, p-Akt (Ser473), cyclin D1, Bcl-2, vimentin, and Snail were significantly increased, and the expressions of Bax and E-cadherin were markedly decreased in PTC tissues compared with the adjacent noncancer tissues (NCT). Overall, these findings suggested that miR-101 confers anticarcinogenic effects in PTC by targeting CXCL12 signaling.

MATERIALS AND METHODS

Clinical Specimens

A total of 82 pairs of formalin fixed paraffin-embedded PTC tissue specimens and the adjacent NCT were obtained from PTC patients who underwent surgical resection at the Department of Oncology, Henan Provincial People's Hospital (Zhengzhou, P.R. China), between 2010 and 2016 for immunohistochemistry (IHC) assay. Clinicopathological characteristics of 82 PTC patients are summarized in Table 1. Another 20 pairs of fresh PTC and

Table 1. Correlation Between Clinicopathological Features and miR-101 Expression in the Papillary Thyroid Carcinoma (PTC) Cases

		miR-101 Level		
Variables	No. of Cases ($n=82$)	High ($n=38$)	Low ($n=44$)	p Value
Age (year)				0.376
<45	41	21	20	
≥45	41	17	24	
Sex				0.056
Male	34	20	14	
Female	48	18	30	
Tumor size (cm)				0.725
≤2	64	29	35	
>2	18	9	9	
Capsule invasion				0.324
No	32	17	15	
Yes	50	21	29	
Multifocality				0.402
No	34	16	18	
Yes	48	22	26	
TNM stage				0.014*
I/II	51	26	25	
III/IV	31	12	19	
Primary tumor				0.008*
T1/T2	42	24	18	
T3/T4	40	14	26	
Lymph node metastasis				0.001*
No	45	25	20	
Yes	37	13	24	
Distant metastasis				0.025*
No	75	36	39	
Yes	7	2	5	

*$p<0.05$

NCT tissues were immediately collected and stored in liquid nitrogen or −80°C for quantitative real-time PCR (qPCR) and Western blot assays. None of the patients had received radio- or chemotherapy prior to surgery. Tissue samples were classified according to World Health Organization classification (2017 Ver.). This study was approved by the Institutional Review Board of the Henan Provincial People's Hospital, and written informed consents were obtained from each patient.

Cell Culture and Transfection

The human benign thyroid follicular epithelial cell line Nthy-ori 3-1 and PTC cell lines (NPA, K1, B-CPAP, TPC-1, and GLAG-66) were purchased from American Type Culture Collection (Manassas, VA, USA). Nthy-ori 3-1, NPA, B-CPAP, and TPC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). K1 and GLAG-66 cells were grown in DMEM/Ham's F12/MCDB (2:1:1; Gibco) supplemented with 10% FBS (Gibco). All cell lines were cultured in a humidified atmosphere at 37°C with 5% CO_2.

Cell Transfection

miR-101 mimics and negative control miR-NC were synthesized by Ribobio (Guangzhou, P.R. China). Plasmids pcDNA3.1 and pcDNA3.1-CXCL12 were procured from GenePharma (Shanghai, P.R. China). After confluence reached 70%–80%, B-CPAP and TPC-1 cells were transfected with miR-NC, miR-101 mimics, or miR-101 mimics + pcDNA3.1-CXCL12 plasmid using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

RNA Isolation and qPCR

Total RNA was isolated from tissue specimens and cells using RNeasy mini kit (Qiagen, Hilden, Germany) according to the protocols. cDNA was synthesized using miScript Reverse Transcription Kit (Qiagen) or the PrimeScript RT reagent kit (Takara, Dalian, P.R. China). Amplification was performed on an ABI 7900 real-time PCR system (Applied Biosystems, White Plains, NY, USA) using the SYBR Premix Ex Taq™ (Takara). The levels of miRNA and mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method, with U6 small nuclear RNA (snRNA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal controls, respectively. Primers were as follows: for miR-101, 5′-CGCTTCGGCAGCACATATA C-3′ (forward) and 5′-TTCACGAATTTGCGTGTCAT-3′ (reverse); for U6, 5′-AGGAAGGCGGACATATTAGTC CCT--3′ (forward) and 5′-AGACGATAGTTGGGTCCC GGC-3′ (reverse); for CXCL12, 5′-GCCATGAACGCC AAGGTC-3′ (forward) and 5′-CGAGTGGGTCTAGCG GAAAG-3′ (reverse); for GAPDH, 5′-AACGGATTTG GTCGTATTG-3′ (forward) and 5′-GGAAGATGGTGA TGGGATT-3′ (reverse).

Dual-Luciferase Reporter Assay

The wild-type (WT) or the mutant (MUT) 3′-UTR of CXCL12 in the putative miR-101 binding sites was inserted downstream of the firefly luciferase gene in the pGL3 vector (Promega, Madison, WI, USA). For luciferase assay, B-CPAP and TPC-1 cells were seeded into 24-well plates and then cotransfected with 50 ng of pGL3-CXCL12-3′-UTR-WT or pGL3-CXCL12-3′-UTR-MUT and 100 nM of miR-NC or miR-101 mimics using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, the luciferase activities were measured with the Dual-Luciferase Reporter System (Promega). *Renilla* luciferase was cotransfected as a control for normalization.

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation was measured using CCK-8 kit (Beyotime, Shanghai, P.R. China) as per the manufacturer's instructions. In brief, B-CPAP and TPC-1 cells were seeded into 96-well plates at a density of 1×10^3 cells/well and then transfected with the indicated oligonucleotides. After transfection for 1, 2, 3, or 4 days, CCK-8 solution (10 µl) was added to each well, and the absorbance at 450 nm was measured under a multiwell spectrophotometer (Bio-Rad, Richmond, CA, USA) after incubation for 2 h at 37°C.

Colony Formation Assay

B-CPAP and TPC-1 cells (1×10^5) transfected with miR-NC, miR-101 mimics, or miR-101 mimics + pcDNA3.1-CXCL12 plasmid were seeded in six-well plates precoated by 1% agar (Sigma-Aldrich, St. Louis, MO, USA) and then cultured for 14 days. After 70% ethanol fixation, the colonies were stained with 0.5% crystal violet (Sigma-Aldrich) and counted under a microscope (Carl-Zeiss, Oberkochen, Germany). Five random fields of each well were selected to determine the total number of colonies.

Motility Assays

Cell migration and invasion were assessed using Transwell chambers. For migration assay, 5×10^3 B-CPAP and TPC-1 cells transfected with the indicated oligonucleotides were seeded into the upper chamber of 8-µm pore size Transwells (BD Biosciences, Franklin Lakes, NJ, USA). For invasion assay, 1×10^4 cells were added to the upper chamber of 8-µm pore size Tranwells precoated with Matrigel (BD Biosciences). The medium containing CXCL12 was added to the lower chamber,

serving as a chemoattractant. After 24 h of incubation, the cells that had not migrated or invaded through the pores were carefully removed. The filters were then fixed with methanol and stained by 0.5% crystal violet (Sigma-Aldrich). Five random fields per chamber were counted using an inverted microscope (Carl-Zeiss).

Apoptosis Assay by Flow Cytometry

Cell apoptosis was measured using Annexin V–fluorescein isothiocyanate (FITC) Apoptosis Detection Kit I (BD Biosciences) following the manufacturer's protocol. At 48 h after transfection of miR-NC, miR-101 mimics, or miR-101 mimics + pcDNA3.1-CXCL12 plasmid, B-CPAP and TPC-1 cells were harvested, centrifuged, and resuspended in binding buffer. Then 10 μl of ready-to-use Annexin V–FITC was added into the mixture, incubated at 37°C for 15 min, and counterstained with 5 μl of propidium iodide (PI; Sigma-Aldrich) in the dark for 30 min. Annexin V–FITC and PI fluorescence were detected by BD FACSCalibur flow cytometry (BD Biosciences). Results were analyzed by CellQuest software (BD Biosciences).

Western Blotting

Proteins were extracted from fresh tissues and cells, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA), and subjected to immunoblot analyses. Blotting was performed with primary antibodies against CXCL12, Akt, p-Akt (Ser473), Bax, Bcl-2 (all from Cell Signaling Technology, Danvers, MA, USA), cyclin D1, E-cadherin (both from Abcam, Cambridge, UK), vimentin, Snail (both from Abnova, Taiwan, P.R. China), and β-actin (Sigma-Aldrich), followed by horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich). Bands were visualized using the enhanced chemiluminescence kit (Santa Cruz Biotechnology, Dallas, TX, USA).

IHC Staining

Paraffin-embedded PTC and NCT tissues were sectioned at 5-μm thickness. The sections were deparaffinized, rehydrated, and quenched with 3% hydrogen peroxide for 15 min at room temperature. After immersing in blocking solution (5% normal horse serum and 1% goat serum in PBS) for 10 min, the slides were incubated for 1 h at 37°C with primary antibodies targeting CXCL12, p-Akt (Ser473), cyclin D1, Bax, Bcl-2, E-cadherin, vimentin, and Snail. After incubation, the sections were washed with PBS for 10 min, and anti-mouse or anti-rabbit biotinylated secondary antibodies were applied. After rinsing, the avidin–biotin–peroxidase complex (Maixin Biotech., Fuzhou, P.R. China) was used to incubate with the sections, followed by extensive washing steps. The sections were incubated with diaminobenzidine (Maixin Biotech.) and counterstained with commercial hematoxylin (Maixin Biotech.). After dehydration and transparency, the slides were mounted and observed under light microscopy (Carl-Zeiss).

Statistical Analysis

Data are expressed as mean ± SD from three independent experiments. Statistical differences were compared using Student's *t*-test and ANOVA. The relationship of miR-101 and CXCL12 level was determined by Pearson's correlation analysis. Correlation between miR-101 expression and the clinical characteristics was analyzed by chi-square test. All p values were two sided and obtained using the SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

miR-101 Lowly Expresses in PTC Cells and Inversely Correlates With CXCL12 Expression

To investigate the biological roles of miR-101 in PTC, we first measured the differential expressions of miR-101 in human benign thyroid follicular epithelial cell line Nthy-ori3-1 and PTC cell lines (NPA, K1, B-CPAP, TPC-1, and GLAG-66) by qPCR assay. As shown in Figure 1A, miR-101 was significantly downregulated in all five PTC cell lines compared with Nthy-ori 3-1 cells. We next detected the mRNA and protein levels of CXCL12 by qPCR and Western blot assays. Both the mRNA (Fig. 1B) and protein (Fig. 1C) levels of CXCL12 in PTC cells were much higher than those in Nthy-ori 3-1 cells. The B-CPAP and TPC-1 cell lines that had the lowest expression of miR-101 and the highest level of CXCL12 were selected for the following experiments. Moreover, miR-101 expression was inversely associated with CXCL12 mRNA and protein levels in PTC cells (Fig. 1D and E). These data implied that miR-101 negatively associates with CXCL12, and it may play important roles in PTC.

miR-101 Directly Targets CXCL12 in PTC Cells

Based on the inverse correlation between miR-101 and CXCL12 expression, we probed whether miR-101 directly targets CXCL12 in PTC cells using the predictive program. Intriguingly, among common predicted targets of miR-101, there was a putative binding site of miR-101 in the 3′-UTR of CXCL12 (Fig. 2A). Moreover, dual-luciferase reporter activity assay showed that miR-101 could suppress luciferase activity of the reporter plasmid with CXCL12 3′-UTR-WT but did not

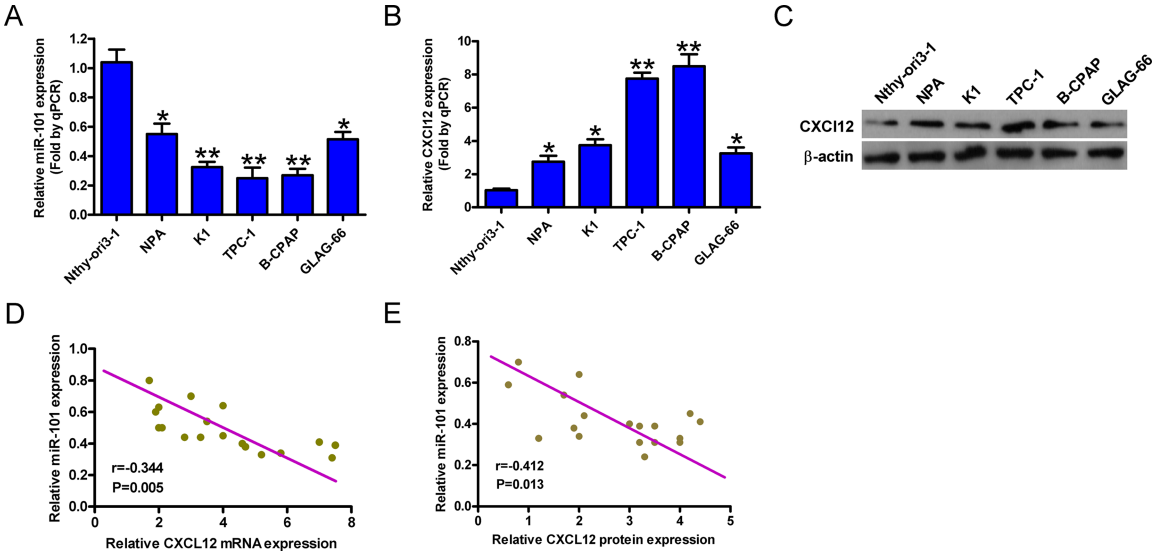

Figure 1. The levels of microRNA-101 (miR-101) and chemokine (C-X-C motif) ligand 12 (CXCL12) in papillary thyroid carcinoma (PTC) cell lines. (A) Quantitative real-time PCR (qPCR) assay was performed to measure the levels of miR-101 in the benign human thyroid follicular epithelial cell line Nthy-ori 3-1 and PTC cell lines (NPA, K1, B-CPAP, TPC-1, and GLAG-66). U6 was used as internal control. (B) mRNA and (C) protein expressions of CXCL12 were detected by qPCR and Western blot assays in Nthy-ori 3-1 and PTC cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as the endogenous controls, respectively. The correlations between miR-101 expression and the (D) mRNA or (E) protein levels of CXCL12 were determined by Pearson's correlation analyses. Data are expressed as means ± SD of three separate experiments. *$p<0.05$, **$p<0.01$ compared with Nthy-ori 3-1 group.

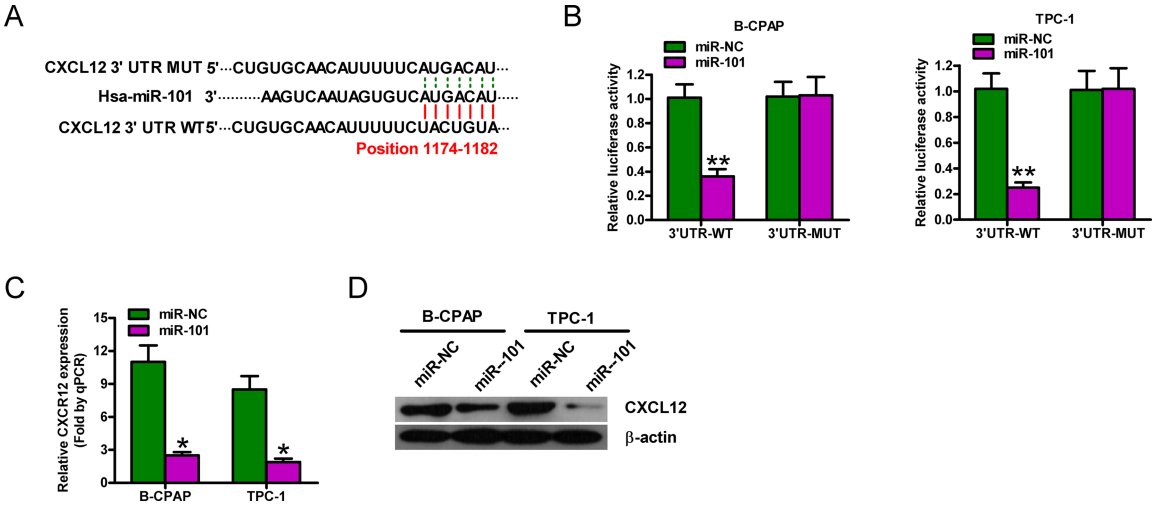

Figure 2. CXCL12 was a direct target of miR-101 in PTC cells. (A) Binding sequence of miR-101 in CXCL12 3′-UTR was predicted by Targetscan database. (B) Relative luciferase activities of the B-CPAP and TPC-1 cells that were cotransfected with wild-type (WT) or mutant (MUT) 3′-UTR of CXCL12 and miR-101 mimics or miR-NC. Data were normalized to those from cells cotransfected with miR-NC and WT 3′-UTR of CXCL12 plasmid. The (C) mRNA and (D) protein levels of CXCL12 in B-CPAP and TPC-1 cells after transfection with miR-NC or miR-101 mimics were analyzed by qPCR and Western blot assays. GAPDH and β-actin were used as the endogenous controls, respectively. Data are expressed as means ± SD of three separate experiments. *$p<0.05$, **$p<0.01$ compared with miR-NC group.

influence the activity of the reporter fused to CXCL12 3′-UTR-MUT in B-CPAP and TPC-1 cells (Fig. 2B). qPCR and Western blot analyses further confirmed that miR-101 repressed CXCL12 mRNA (Fig. 2C) and protein (Fig. 2D) expressions in B-CPAP and TPC-1 cells compared with the miR-NC-treated cells. These results indicated that CXCL12 is a direct target of miR-101 in PTC cells.

Restoration of CXCL12 Counteracts miR-101-Imposed Suppressive Effects on the Proliferation and Apoptosis Resistance in PTC Cells

We next explored whether CXCL12 is a functional target of miR-101 in PTC. As shown in Figure 3A, miR-101 significantly reduced the viability of B-CPAP and TPC-1 cells compared with miR-NC-transfected cells. However, cotransfection with pcDNA3.1-CXCL12 plasmid markedly rescued miR-101-exerted inhibition of PTC cell viability. Colony formation assay showed that the reductive colony numbers caused by miR-101 was restored by CXCL12 overexpression in B-CPAP and TPC-1 cells (Fig. 3B and C). Analogously, miR-101 increased the apoptosis of B-CPAP and TPC-1 cells, whereas CXCL12 overexpression attenuated miR-101-induced cell apoptosis (Fig. 3D and E). To identify the molecular mechanisms by which miR-101 exerts suppressive effects on PTC cell proliferation and apoptosis resistance, we examined the expressions of proliferative- and apoptotic-associated proteins by Western blotting. As depicted in Figure 3F, in comparison with miR-NC-transfected cells, miR-101 led to substantial decrease in CXCL12, p-Akt (Ser473), cyclin D1, and Bcl-2 levels, accompanied by a significant increase in Bax expression in B-CPAP and TPC-1 cells. However, cotransfection with pcDNA3.1-CXCL12 plasmid reversed the expressions of the above-mentioned proteins. These results suggested that CXCL12 is a functional target of miR-101 in PTC cell proliferation and apoptosis escape.

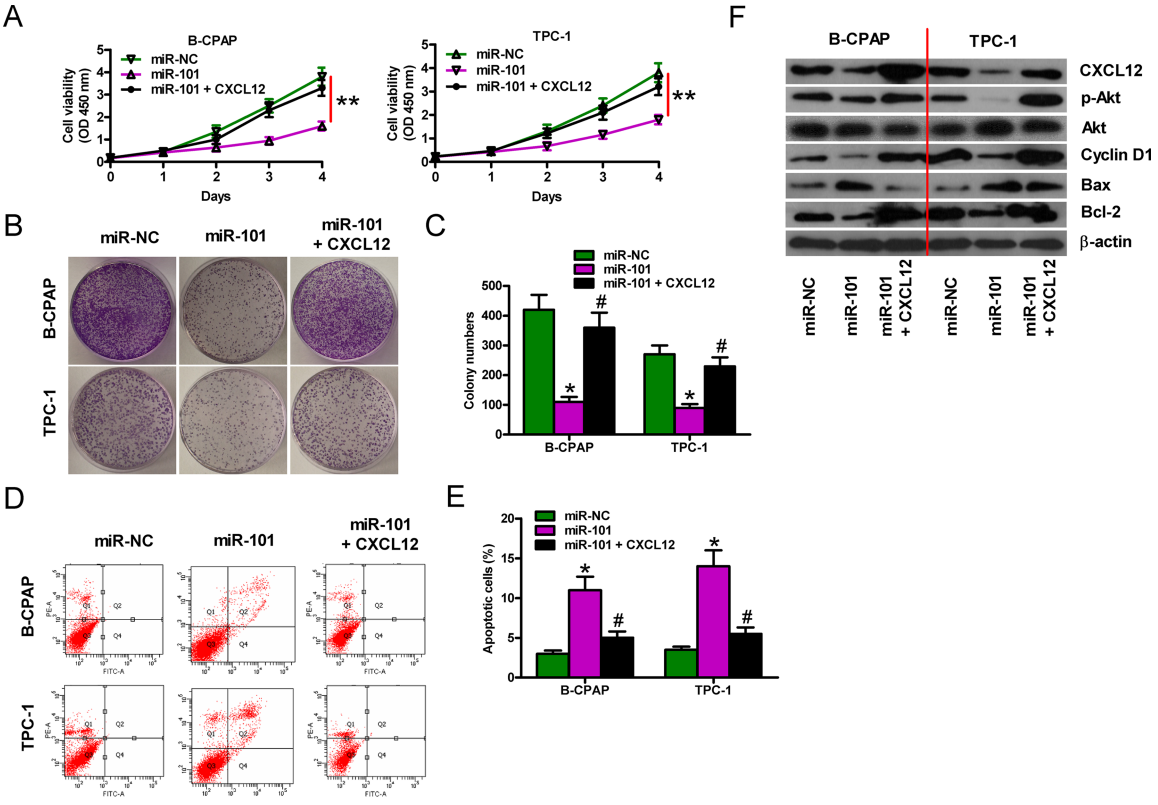

Figure 3. CXCL12 restoration counteracted the suppressive effects of miR-101 on the proliferation and apoptosis resistance of PTC cells. B-CPAP and TPC-1 cells were transfected with miR-NC or miR-101 mimics or miR-101 mimics + CXCL12-overexpressing plasmid. (A) Cell viability was assessed by cell counting kit-8 (CCK-8) assay at 1, 2, 3, and 4 days after transfection. (B) Cell proliferation was measured by colony formation assays. (C) The colony number was calculated. (D) Cell apoptosis was analyzed by flow cytometry. (E) The apoptotic rate was calculated. (F) The levels of CXCL12, Akt, p-Akt (Ser473), cyclin D1, Bcl-2, and Bax were determined by Western blot assays. β-Actin was used as endogenous control. Data are expressed as means ± SD of three separate experiments. $^{*}p < 0.05$ compared with miR-NC group; $^{**}p < 0.01$ and $^{\#}p < 0.05$ compared with miR-101 group.

CXCL12 Attenuates miR-101-Decreased Migration, Invasion, and EMT of PTC Cells

To uncover whether the effects of miR-101 on PTC cell migration and invasion are mediated by CXCL12, B-CPAP and TPC-1 cells were transfected with miR-NC or miR-101 mimics or miR-101 mimics + pcDNA3.1-CXCL12 plasmid. Transwell assay showed that miR-101 markedly reduced the migration of B-CPAP and TPC-1 cells, and CXCL12 overexpression attenuated the reductive migratory cells caused by miR-101 (Fig. 4A and B). Also, the invasion of B-CPAP and TPC-1 cells was abated by miR-101, which was neutralized by CXCL12 overexpression (Fig. 4C and D). Molecularly, downregulation of the epithelial cell marker E-cadherin and simultaneous upregulation of the mesenchymal markers vimentin and Snail were observed in miR-101-transfected B-CPAP and TPC-1 cells. However, cotransfection with pcDNA3.1-CXCL12 plasmid reversed the above-mentioned protein expressions (Fig. 4E). These results demonstrated that CXCL12 is a downstream functional target of miR-101 in motility of PTC cells by regulating the EMT.

miR-101 Is Lowly Expressed and Inversely Correlated With CXCL12 Expression in PTC Tissues

To explore the differential expressions of miR-101 and CXCL12 in PTC and NCT tissues, qPCR assays were performed. miR-101 expression was much lower in PTC tissues than that in NCT tissues (Fig. 5A). Downregulation of miR-101 was negatively associated with advanced histological stages and metastasis (Table 1). Both mRNA and protein levels of CXCL12 were significantly high in PTC tissues compared with NCT tissues (Fig. 5B and C). An inverse correlation between miR-101 level and CXCL12 mRNA expression existed in PTC specimens (Fig. 5D). IHC results revealed high levels of CXCL12, cyclin D1, Bcl-2, vimentin, and Snail and low levels of Bax and E-cadherin in PTC tissues compared with the corresponding NCT tissues (Fig. 5E). These findings stated that miR-101 expression is negatively associated with the levels of CXCL12 and the related tumor-promoting proteins in PTC tissues, suggesting miR-101 as a tumor suppressor in PTC.

DISCUSSION

In this study, we demonstrated the tumor-suppressive roles of miR-101 in PTC (Fig. 6). Key findings were as follows. First, downregulation of miR-101 and upregulation of CXCL12 and inverse correlation of miR-101 and CXCL12 expressions were observed in PTC cells and tissues. Second, miR-101 directly targeted CXCL12 in PTC cells. Third, miR-101 repressed proliferation, migration, and invasion and enhanced apoptosis of PTC cells, whereas CXCL12 overexpression attenuated the above effects of miR-101. Fourth, miR-101 significantly decreased the expressions of p-Akt, cyclin D1, Bcl-2, vimentin, and Snail, but increased the levels of Bax and

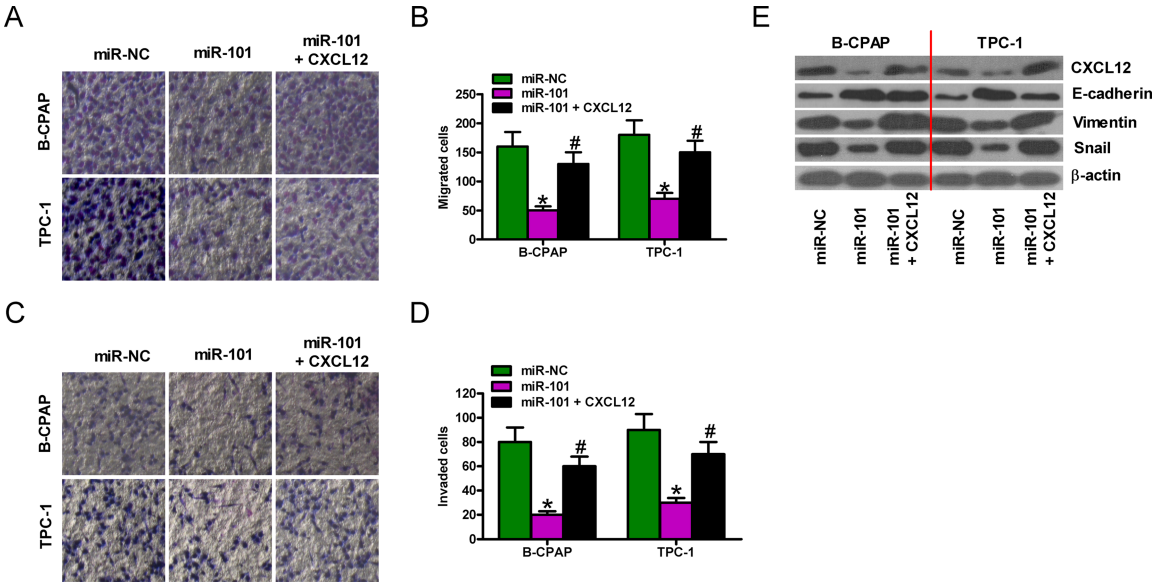

Figure 4. Ectopic expression of CXCL12 attenuated the inhibitory effects of miR-101 on migration and invasion of PTC cells. B-CPAP and TPC-1 cells were transfected with miR-NC or miR-101 mimics or miR-101 mimics + CXCL12-overexpressing plasmid. (A, C) Transwell assays were performed to determine the migration (A) and invasion (C) of cells. (B, D) The migrated (B) and invaded (D) cells were calculated. (E) Expressions of CXCL12, E-cadherin, vimentin, and Snail were analyzed by Western blot assays. β-Actin was used as endogenous control. Data are expressed as means ± SD of three separate experiments. *$p < 0.05$ compared with miR-NC group; #$p < 0.05$ compared with miR-101 group.

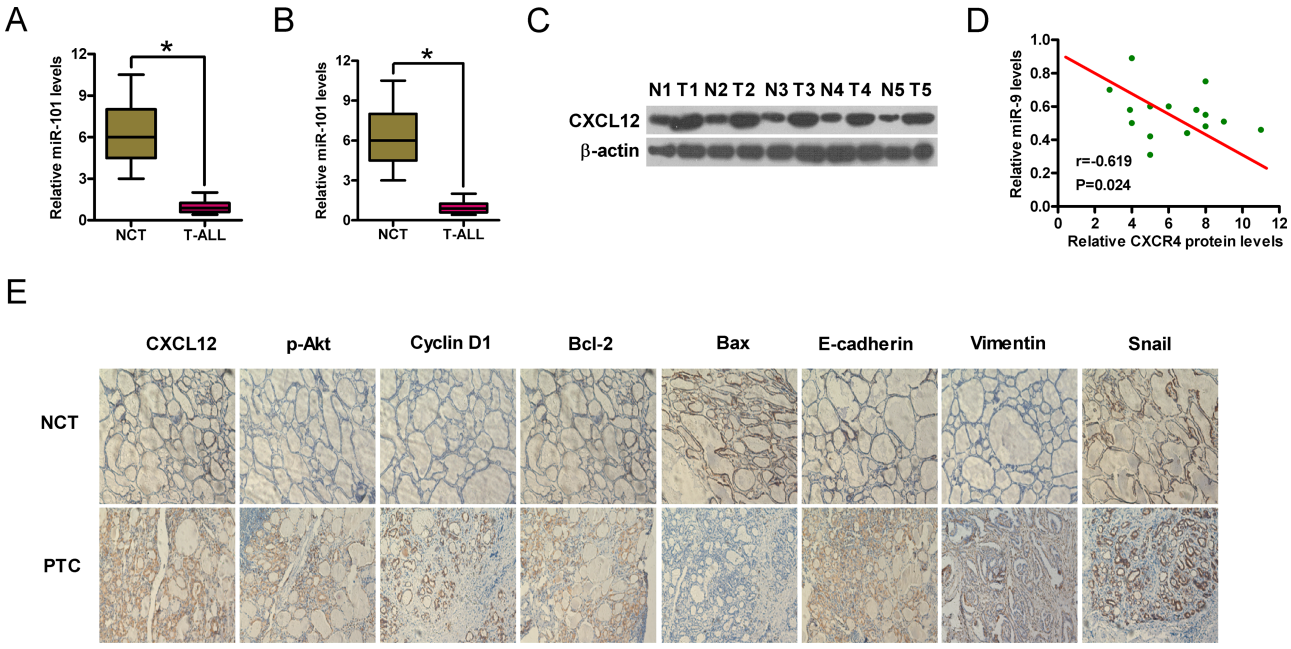

Figure 5. miR-101 and CXCL12 expressions in PTC tissues. (A) qPCR analyses of miR-101 expression and (B) mRNA level of CXCL12 in PTC tissues ($n=20$) and in NCT tissues ($n=20$). U6 and GAPDH were used as the internal controls, respectively. (C) Representative Western blot results of CXCL12 in five pairs of PTC and NCT tissues. β-Actin was used as endogenous control. (D) The correlation between miR-101 expression and CXCL12 mRNA level is shown. (E) IHC results of CXCL12, p-Akt (Ser473), cyclin D1, Bax, Bcl-2, E-cadherin, vimentin, and Snail in PTC and NCT tissues. Data are expressed as means ± SD of three separate experiments. *$p<0.05$ compared with adjacent NCT. NCT, noncancer tissues.

E-cadherin, which were reversed by CXCL12 overexpression in PTC cells. Last, a low level of miR-101 in PTC tissues was negatively associated with several clinical characteristics, including advanced histological stages, lymph node metastasis, and distant metastasis. Overall, these results suggested that miR-101 exerts its suppressive effects on PTC cells by targeting CXCL12/Akt or Snail signaling pathways.

PTC is a well-differentiated thyroid malignancy mostly presenting an indolent clinical behavior and a good prognosis[3,4]. However, 10%–15% of PTC patients often have spread to the cervical lymph nodes and have unfavorable outcomes[4,5]. The severe invasion, lymph node metastasis, and distant metastasis are the leading causes of the high morbidity and mortality in PTC[33]. Accumulating evidence has indicated that miRNAs play crucial roles in tumor development and progression[10,11]. Therefore, it is important to identify tumor-related miRNAs and their targets to improve the treatment of PTC.

Aberrant expression of miRNAs was observed in PTC and was involved in the initiation and progression of PTC[34]. miR-613 has been shown to inhibit the proliferation, migration, and invasion of PTC cells by regulating SphK2 expression[35]. miR-148a significantly suppresses PTC cell proliferation and motility in vitro, and reduces tumor growth in vivo by repressing CDK8 expression[36].

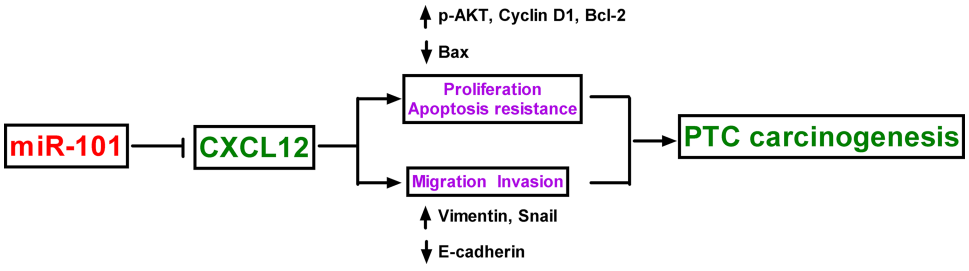

Figure 6. Proposed mechanisms of miR-101 involved in the suppression of PTC carcinogenesis. miR-101 reduced the proliferation, migration, and invasion and induced apoptosis in PTC cells by directly targeting CXCL12 and subsequently regulating the expression of its downstream molecules.

Zhou et al.[37] demonstrated that let-7a abolishes the motility of PTC cells and tumor growth by targeting AKT2. Liu et al.[38] identified that 248 miRNAs are strikingly deregulated in PTC tissues, including miR-101. miR-101 is a well-known tumor suppressor that frequently downregulates and inhibits the progression of multiple cancers, including PTC[12–20]. miR-101 represses the proliferation, migration, and invasion of PTC cells by targeting Rac1[17,18]. Zhao et al.[19] reported that miR-101 reduces PTC cell proliferation, apoptosis resistance, migration, and invasion in vitro and suppresses tumor growth and metastasis in vivo by targeting USP22. miR-101 was able to sensitize PTC cells to TRAIL treatment in vitro and in vivo through driving TRAIL-induced mitochondrial apoptosis by targeting c-met and MCL-1[20]. Consistently, in this study, we demonstrated that miR-101 was pronouncedly downregulated in PTC tissues and cell lines. We showed that miR-101 reduced cell proliferation, impaired migration and invasion, and induced apoptosis of PTC cells. Molecularly, miR-101 markedly decreased the expression of p-Akt, cyclin D1, Bcl-2, vimentin, and Snail and enhanced the level of Bax and E-cadherin. These results suggested that miR-101 is a key tumor suppressor in PTC.

CXCL12 is constitutively expressed in the bones, lymph nodes, liver, lungs, and other organs[39]. CXCL12 binds to its receptor, CXCR4 and/or CXCR7, to activate several key signaling pathways, such as PI3K/Akt, ERK, and JNK pathways, contributing to cell survival and motility[23,24,40]. Increasing evidence suggests that altered expression of CXCL12 contributes to the development and progression of various cancers, including PTC[25–32]. The SDF-1/CXCR4 axis may play positive roles in pancreatic cancer by regulating tumor cell migration and angiogenesis[25]. SDF-1α stimulates the proliferation of glioblastoma cells through the activation of extracellular signal-regulated kinases 1/2 and Akt signaling[26]. SDF-1 increases the invasiveness of breast cancer cells and correlates with node involvement and survival in breast cancer patients[28]. CXCL12 was exclusively expressed in PTC tissues but absent in other types of thyroid malignancies and benign lesions[41]. CXCL12 and its receptors are implicated in PTC carcinogenesis and may be prognostic and/or diagnostic markers for PTC[29–32]. CXCL12 dramatically enhances the invasive ability of B-CPAP cells[32]. Moreover, CXCL12/CXCR4 axis facilitates the EMT process, as evidenced by a decreased level of E-cadherin and increased expressions of N-cadherin and vimentin[32]. In the present study, we found that CXCL12 was remarkably increased in PTC cells and tissues, simultaneously with the upregulation of p-Akt (Ser473), cyclin D1, Bcl-2, vimentin, and Snail and downregulation of Bax and E-cadherin. CXCL12 overexpression promoted the proliferation, migration, and invasion and inhibited apoptosis in PTC cells in vitro. These data demonstrated that CXCL12 is a positive mediator in PTC carcinogenesis.

Previously, CXCL12 has been identified as a direct target of several miRNAs, such as miR-1[42], miR-448[43], miR-454[44], miR-137[45], and miR-101[46], in various cancers. Dong et al.[45] reported that CXCL12 is increased in PTC tissues, and CXCL12 depletion mimics the repressive effects of miR-137, and upregulation of CXCL12 partially reverses the effects of miR-137 on PTC cells. Recently, miR-101 reduces the ability of cancer-associated fibroblasts to stimulate proliferation, sphere formation, migration, and invasion, and apoptosis evasion in lung cancer cells by targeting CXCL12[46]. Similarly, we illuminated that CXCL12 was a direct target of miR-101 in PTC cells, and the reduced proliferation, migration, and invasion and the increased apoptosis in PTC cells caused by miR-101 were neutralized by CXCL12 overexpression, indicating that miR-101 exerts its tumor-suppressive effects in PTC by targeting CXCL12.

In conclusion, we found that miR-101 is markedly downregulated and inversely associated with CXCL12 expression in PTC tissues and cells. Clinically, miR-101 downregulation is positively associated with advanced histological stages and lymph node and distant metastases. Functionally, miR-101 suppresses the proliferation, apoptosis resistance, invasion, and migration of PTC cells by directly targeting CXCL12. Molecularly, the downstream effectors of CXCL12, including p-Akt, cyclin D1, Bax, Bcl-2, E-cadherin, vimentin, and Snail, are highly involved in miR-101-exerted inhibitory effects on PTC. Overall, these results imply that miR-101 hinders the PTC carcinogenesis by targeting CXCL12-mediated Akt and Snail signaling pathways, and miR-101/CXCL12/Akt or Snail axis is a potential target for PTC treatment.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

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