Cadherin-16 inhibits thyroid carcinoma cell proliferation and invasion

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Abstract. Cadherin-16 (CDH16), a member of the cadherin family of adhesion molecules, serves an important role in the formation and maintenance of the thyroid follicular lumen. Decreased expression of CDH16 has been reported to be associated with tumor stage in papillary thyroid cancer (PTC); however, previous analyses have been limited and the biological role of CDH16 in different subtypes of TC is unknown. To investigate the role of CDH16 in the occurrence and development of TC, bioinformatic analysis of three TC subtypes (PTC, follicular cell-derived TC and anaplastic TC) was performed using an extended data set from the Gene Expression Omnibus database, with additional confirmation using data from The Cancer Genome Atlas, as well as biopsies from 35 patients with PTC and TC or follicular cell lines. According to the dataset analysis, CDH16 was downregulated in PTC and follicular cell-derived and anaplastic TC; the downregulation in PTC was independent of DNA copy number variation. Furthermore, low expression levels of CDH16 were significantly correlated with tumor size, lymph node metastasis status and disease stage in 35 patients with PTC. Gene Set Enrichment Analysis suggested that CDH16 participated in DNA replication and cell adhesion pathways. To evaluate CDH16 activity, CDH16 was overexpressed in TC-derived BCPAP cells. CDH16 overexpression inhibited cell proliferation, migration and invasion and induced apoptosis by downregulating proteins associated with DNA replication and cell adhesion. These results support the identification of CDH16 as a valuable target for TC prognosis and therapy and, to the best of our knowledge, represent the first direct demonstration of its mechanistic role in TC.

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Introduction

Thyroid carcinoma (TC) is the most common endocrine malignancy (1), with increasing incidence worldwide (2-4). Epidemiological data from China show that the age standardized incidence rate of thyroid cancer increased from $3.21/10^5$ in 2005 to $9.61/10^5$ in 2015, with an annual increasing incidence rate of 12.4% (5). There are four primary types of TC according to the cellular derivation and state of differentiation (6): Papillary thyroid cancer (PTC), follicular thyroid cancer (FTC) and anaplastic thyroid cancer (ATC) arise from follicular cells of the thyroid, while medullary thyroid cancer (MTC) arises from parafollicular C cells of the thyroid. Although PTC and FTC, the most common forms of TC, usually have good prognoses (7,8), ~10% of patients suffer disease recurrence and metastasis following treatment (9). Furthermore, MTC and ATC have worse prognoses due to their aggressive behavior (10), with a 1-year survival rate of 5-30% for ATC (11). A major challenge in treatment of TC is failure to respond to radiotherapy and chemotherapy (8,12). Therefore, it is urgent to identify new biomarkers and therapeutic targets.

Cadherins (CDHs) are members of a family of homozygous and Ca^{2+}-dependent cell adhesion glycoproteins, which perform essential roles in embryonic development, normal cell function and tissue integrity preservation by mediating cell-cell adhesion (13). Abnormal expression of CDHs has been reported in carcinogenesis and is associated with tumor initiation and progression (14). Notably, CDH16 has been implicated in differentiation of the kidney (15,16) and thyroid (17-19). Furthermore, decreased CDH16 has been observed in renal cell carcinoma (16) and studies suggest that CDH16 may be downregulated in TC (20,21). In one study (21), downregulation of CDH16 was shown to be correlated with unfavorable clinicopathological features of PTC and was inversely associated with expression of cancer-associated genes; however, the analysis did not distinguish between TC subtypes and did not consider the potential contribution of gene copy number variation (CNV). Furthermore, bioinformatic analysis was limited to a single cohort in The Cancer Genome Atlas (TCGA) database and direct expression analysis was limited to a single 16 patient cohort. Though the results of the previous studies (20,21) are consistent with a tumor suppressor role of CDH16, the biological activity of CDH16 in TC has not been directly demonstrated and its role in TC remains hypothetical.

In the present study, the expression and CNV of CDH16 in TC was investigated. Functional assays were also performed to probe its role and molecular mechanism in TC. These studies provide a molecular basis for the role of CDH16 in TC, as well as confirming the potential of CDH16 as a target for TC prognosis and treatment.

Materials and methods

Database analysis. mRNA expression and DNA CN of CDH16 in TC were investigated using the Oncomine 4.5 database (oncomine.org), an integrated data mining platform for collecting, analyzing and delivering cancer transcriptome data (22). The present study analysis focused on four TC studies, including He (accession no. GSE3467), Vasko (accession no. GSE6004), Giordano (accession no. GSE27155) and TCGA thyroid (23-25). The CDH16 mRNA expression and DNA CN were assessed between TC and normal thyroid tissue. $P<0.05$ was considered to indicate a statistically significant difference.

LinkedOmics database (linkedomics.org/login.php), a publicly available portal analyzing multi-omics data from 32 types of cancer in TCGA (26), was used to visualize genes that were co-expressed with CDH16 and perform KEGG pathway analysis of these genes by Gene Set Enrichment Analysis (GSEA).

Tissue specimens and cell culture. A total of 35 paired PTC surgical and corresponding adjacent normal thyroid specimens were obtained from Tangshan Gongren Hospital and Tangshan Renmin Hospital between January 2017 and November 2021. The inclusion criteria were: i) histologically confirmed PTC; ii) voluntary participation in the research; iii) no preoperative radiotherapy, chemotherapy or other treatment for TC. The exclusion criteria were: i) presence of tumor other than TC; ii) heart, lung, liver, kidney or hematopoietic system disease; iii) pregnant or lactating. Patient characteristics are presented in Table I. The samples were stored in liquid nitrogen for reverse transcription-quantitative (RT-q)PCR or fixed in 4% paraformaldehyde solution for 48 h at room temperature and embedded in paraffin for immunohistochemistry (IHC). The present study was approved by the Ethical Committee of Tangshan Gongren Hospital (Hebei, China; approval no. GRYY-LL-2020-104) and all patients provided written informed consent.

Human TC BCPAP and TPC1 cells were obtained from American Type Culture Collection and the human thyroid follicular cell line Nthy-ori3-1 was purchased from Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). BCPAP, TPC1 and Nthy-ori3-1 cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.). All cell lines were cultured in a 5% CO_2 humidified incubator at 37˚C.

IHC staining. Tissue fixation, paraffin embedding, sectioning and dewaxing were performed as previously described (27). Tissue samples were incubated with mouse anti-CDH16 (1:100; cat. no. ab215769; Abcam) overnight at 4˚C. Subsequently, sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (1:2,000; cat. no. ab205719; Abcam) for 30 min at 37˚C. The slides were examined by two blinded senior pathologists from Tangshan Gongren Hospital. Cells within five randomly selected fields of view were counted under a light microscope (magnification, x20) and those with brown or yellow staining on the cell membrane were considered CDH16-positive. The percentage of positive tumor cells was calculated. A threshold of >25% positively stained tumor cells was used to define CDH16 positivity.

RT-qPCR. RNA was isolated from thyroid tissue samples or cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA using cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The prepared cDNA was subjected to RT-qPCR using a SYBR Green PCR Supermix kit (Invitrogen; Thermo Fisher Scientific, Inc.) with the Rotor Gene-3000 instrument (Corbett Research Ltd.). Reactions were conducted in a 20 μl volume with 1 μl cDNA according to the manufacturer's protocol of the SYBR Green PCR Supermix kit. Primer sequences for CDH16 were forward, 5'-CCCTGAGTTCATCACTTCCC-3' and reverse, 5'-AGAGTCTGGCTCCCAATCC-3'. Primer sequences for GAPDH were forward, 5'-GAAAGCCTGCCGGTGACTAA-3' and reverse, 5'-AGGAAAAGCATCACCCGGAG-3'. The PCR protocol was as follows: Initial denaturation at 95˚C for 2 min, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 30 sec. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (28) with GAPDH as a reference gene for normalization.

Western blot analysis. Protein was isolated from cells using RIPA buffer (Beyotime Institute of Biotechnology) with protease inhibitor phenylmethylsulfonyl fluoride (1:200; Beyotime Institute of Biotechnology). Total protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Aliquots of 25 μg protein were electrophoresed on 10% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Membranes were blocked in 5% non-fat milk in TBS containing 0.05% Tween-20 (TBST) for 2 h at room temperature. The membranes were incubated overnight at 4˚C with primary antibodies against CDH16 (1:1,000, cat. no. 15107-1-AP, ProteinTech Group, Inc.), DNA polymerase (POL)D1 (1:1,000, cat. no. 15646-1-AP, ProteinTech Group, Inc), minichromosome maintenance (MCM)6 (1:2,000, cat. no. 13347-2-AP; ProteinTech Group, Inc), claudin (CLDN)1 (1:1,500, cat. no. 13050-1-AP, ProteinTech Group, Inc), intercellular adhesion molecule (ICAM)1 (1:2,000, cat. no. 60299-1-Ig, ProteinTech Group, Inc), syndecan (SDC)4 (1:1,000, cat. no. 11820-1-AP, ProteinTech Group, Inc) or β-actin (1:1,000, cat. no. TA-09; OriGene Technologies, Inc.). The membranes were washed with PBS three times and subsequently probed with anti-rabbit (cat. no. ZB-2301) or anti-mouse horseradish peroxidase-linked secondary antibody (both 1:2500; cat. no. ZB-2305) for 60 min at room temperature and visualized with ECL reagent (cat. no. sc-2048; all OriGene Technologies, Inc.). The protein bands were scanned and quantified by Tanon Gis software v4.2 (Tanon Science and Technology Co., Ltd.). Expression of β-actin served as the loading control.

Table I. Association of CDH16 protein expression with clinicopathological features of 35 patients with papillary thyroid cancer.

		CDH16 expression			
Characteristic	n	+	-	%	P-value
Sex					
Male	10	4	6	60.0	0.471[a]
Female	25	14	11	44.0	
Age, years					
<55	20	11	9	45.0	0.625
≥55	15	7	8	53.3	
Tumor size, cm					
≥2	11	1	10	90.9	0.001
<2	24	17	7	29.2	
Lymph node metastasis					
Present	18	5	13	72.2	0.004
Absent	17	13	4	23.5	
Disease stage					
I, II	28	17	11	39.3	0.041[a]
III, IV	7	1	6	85.7	

[a]P-value calculated by Fisher's exact test. +, CDH16-positive expression; -, CDH16-negative expression.

Gene transfection. A full length CDH16 cDNA clone was chemically synthesized by Sino Biological, Inc. and ligated into pCMV3 vector (Sino Biological, Inc.). Empty pCMV3 vector was used as the negative control. Untransfected cells were used as the blank control group. To introduce the vector, BCPAP cells were seeded in 6-well plates at a density of $5x10^5$ cells per well. After incubation at 37˚C overnight, the degree of cell fusion was 50-70%. The cells were transiently transfected with plasmid carrying CDH16 or negative control plasmid (both 4 μg/well) using TransIntro EL (TransGen Biotech Co., Ltd.) according to the manufacturer's instructions. The effect of pCMV3-CDH16 on CDH16 mRNA and protein expression was analyzed by RT-qPCR and western blotting, as aforementioned, at 48 h post-transfection.

MTT analysis. Cells ($1x10^4$ per well) were seeded in 96-well plates. At 0, 24, 48, 72, and 96 h post-transfection, cell viability was determined by adding 10 μl 5 mg/ml MTT solution (Sigma-Aldrich; Merck KGaA) to each well and incubating the samples for 4 h at 37˚C. The cell culture medium was removed and 150 μl DMSO (Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan crystals. The absorbance at 490 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc.). The experiment was repeated three times.

Flow cytometry analysis. Effect of CDH16 on BCPAP cell apoptosis was determined by flow cytometry. Cells ($5x10^5$ per well) were seeded in 6-well plates. At 48 h post-transfection, the cells were trypsinized using 0.25% trypsin (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 min and collected by centrifugation at 800 x g for 5 min at room temperature. The cells were incubated with 0.5 ml binding buffer and 1.0 μl Annexin V-FITC (Merck KGaA) at room temperature for 15 min and resuspended in fresh 0.5 ml binding buffer containing 5 μl PI. Early apoptosis was measured using a Beckman Coulter FC500 flow cytometer (Beckman Coulter, Inc.) and MXP 2.2 software (Beckman Coulter, Inc.).

Scratch test. Effect of CDH16 on BCPAP cell migration was determined by scratch test. The cells were inoculated on 6-well plates at a density of $5x10^5$ cells/well and incubated in RPMI-1640 (Gibco) containing 10% FBS (Gibco) at 37˚C for 24 h. When confluency reached 80%, the medium was replaced with serum-free RPMI-1640 and the cells were starved for 24 h. A scratch was then drawn with a 200-μl plastic pipette tip and the plates were rinsed with PBS to remove any cells suspended in culture medium after scratching. The gap distance of each scratch wound was photographed at 0 and 48 h after scratching using an light microscope (Olympus Corporation IX71; x40 magnification) and scratch area was measured with ImageJ v1.50 (National Institutes of Health). Wound healing was calculated as a percentage.

Transwell invasion assay. Pre-coated Matrigel chambers (BD Biosciences) were used for the invasion assays according to the manufacturer's instructions. After transfection of pCMV3-CDH16 plasmid and empty pCMV3 plasmid at 37˚C for 48 h, the cells were collected. A total of $1x10^4$ BCPAP cells were seeded into the upper chamber of the Transwell apparatus in serum-free RPMI-1640, while RPMI-1640 supplemented with 10% FBS was added to the lower chamber. After incubation at 37˚C for 24 h, cells adhering to the lower surface were fixed with 100% methanol for 20 min at room temperature and stained with 5% Giemsa solution for 20 min at room temperature. The number of infiltrated cells was calculated under a light microscope (Olympus Corporation IX71) on five randomly selected fields (x100 magnification).

Statistical analysis. All experiments were performed ≥3 times. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc.). The χ^2 or Fisher's exact test was used to analyze differences in CDH16 levels between PTC and normal thyroid tissue in IHC staining. Data are presented as the mean ± standard deviation. The paired t-test was used to compare CDH16 mRNA expression between 35 paired thyroid tissue samples. Pearson correlation test was used in correlation analysis. Single factor analysis of variance (one-way ANOVA) followed by post hoc Fisher's least significant difference test was used to compare >2 samples. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CDH16 is downregulated in human TC tissue and cell lines independent of CNV. To verify that CDH16 is downregulated in TC, CDH16 transcript levels were evaluated in three independent TC studies from GEO. Data from all three studies in the Oncomine database showed that mRNA expression of CDH16 was significantly lower in PTC than in normal thyroid tissue

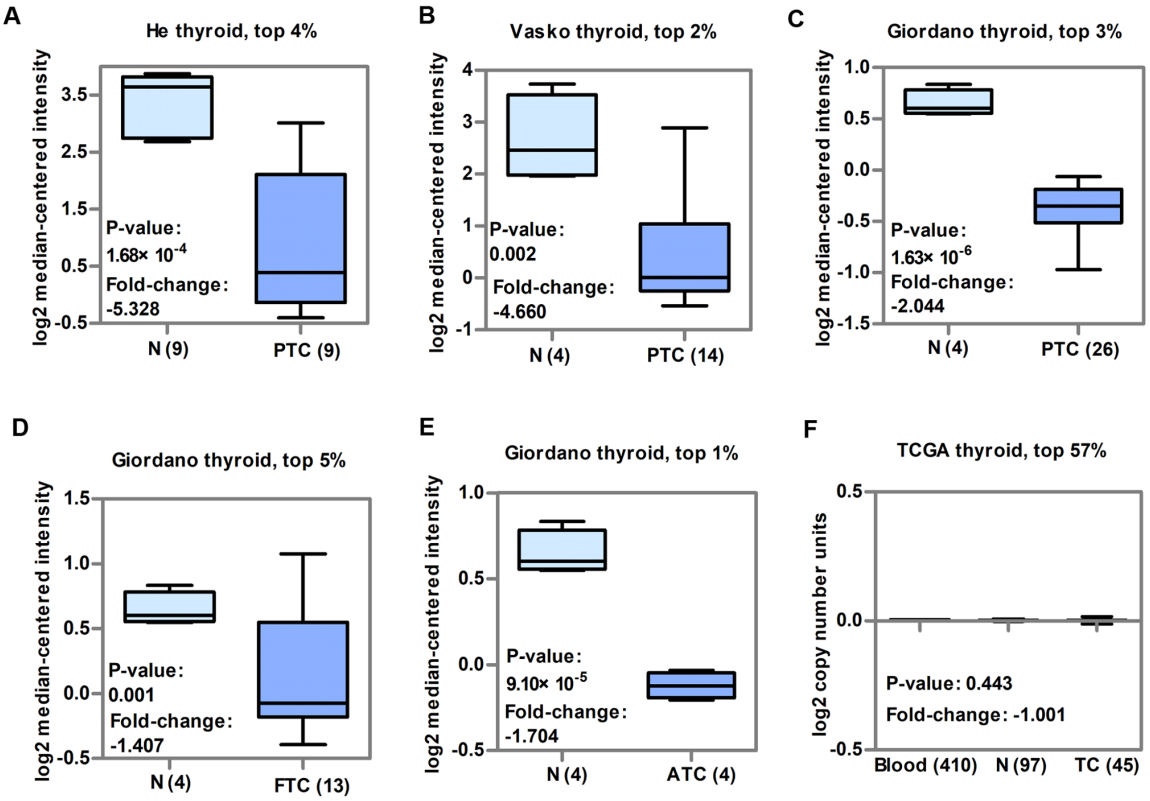

Figure 1. CDH16 is downregulated in TC from three GEO datasets and there is no significant variation in copy number in TCGA dataset. The fold-change, P-value and underexpression gene ranks are based on Oncomine 4.5 analysis. Box plots showing CDH16 mRNA levels in patients with PTC in (A) He, (B) Vasko and (C) Giordano thyroid datasets from GEO. Box plot showing CDH16 mRNA levels in patients with (D) FTC and (E) ATC in the Giordano thyroid dataset. (F) Box plot showing CDH16 copy number in TCGA thyroid dataset. N, normal; CDH, cadherin; TC, thyroid carcinoma; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; ATC, anaplastic thyroid cancer.

(P<0.05; Fig. 1A-C). CDH16 expression was also significantly lower in FTC (P<0.05; Fig. 1D) and ATC (P<0.05; Fig. 1E) than in normal thyroid tissue. Notably, CDH16 ranked in the top 5% of all genes downregulated in each of these studies and the fold difference for PTC was >2. To determine whether decreased expression of CDH16 in PTC may be accounted for by DNA CNV, a dataset from TCGA that has previously been shown to have lower CDH16 expression in PTC (21) compared with matched normal tissue was used. The DNA CN of CDH16 showed no significant difference in blood, normal thyroid or TC tissue (P>0.05; Fig. 1F). Collectively, these findings indicated that CDH16 expression was decreased in TC in three PTC datasets as well as FTC and ATC, and also demonstrated that lower expression of CDH16 in TC was not accounted the result of CNV.

To support these findings, RT-qPCR and IHC were performed for 35 paired thyroid tissue samples collected from patients with PTC. CDH16 mRNA expression was significantly decreased in PTC compared with normal tissue (P<0.05; Fig. 2A). Furthermore, IHC showed that CDH16 was localized to the cell membrane of PTC cells. Of 35 PTC tissue samples, 17 (48.6%) stained negative for CDH16, while only two (5.7%) of the normal thyroid tissue samples stained negative for CDH16 (P<0.05; Fig. 2B). Therefore, decreased expression of CDH16 in PTC was observed at both the mRNA and protein level.

Next, to determine whether downregulation of CDH16 was correlated with the outcome of PTC, the association between CDH16 expression and clinicopathological characteristics of 35 patients with PTC was assessed. CDH16 protein expression was associated with tumor size, lymph node metastasis and pathological stage of PTC but there was no significant association with sex or age (Table I).

To confirm the downregulation of CDH16 in TC, CDH16 levels in TC-derived cell lines (BCPAP and TPC1) and the human thyroid follicular cell line Nthy-ori3-1 were measured. CDH16 expression was lower in TC cell lines, particularly BCPAP cells, both at the mRNA and protein level (P<0.05; Fig. 2C and D). These results were consistent with the aforementioned downregulation of CDH16 in patients with advanced PTC and support the use of BCPAP cells as a model for evaluating CDH16 biological activity.

CDH16 expression is negatively associated with expression of proliferation and invasion pathway-associated genes. To determine the role of CDH16 in TC, genes co-expressed with CDH16 in TC samples from TCGA were investigated using the LinkedOmics database. Volcano plot suggested that the most highly co-expressed genes were polypeptide N-acety lgalactosaminyltransferase 7 (GALNT7), NGFI-A binding protein 2 (NAB2) and UDP-galactose-4-epimerase (GALE), and the most highly inversely co-expressed genes are trefoil factor 3 (TFF3), odontogenic, ameloblast associated (ODAM) and dipeptidyl peptidase like 6 (DPP6) (P<0.05; Fig. 3A). KEGG pathway analysis by GSEA showed that negatively CDH16-associated genes were enriched in DNA replication

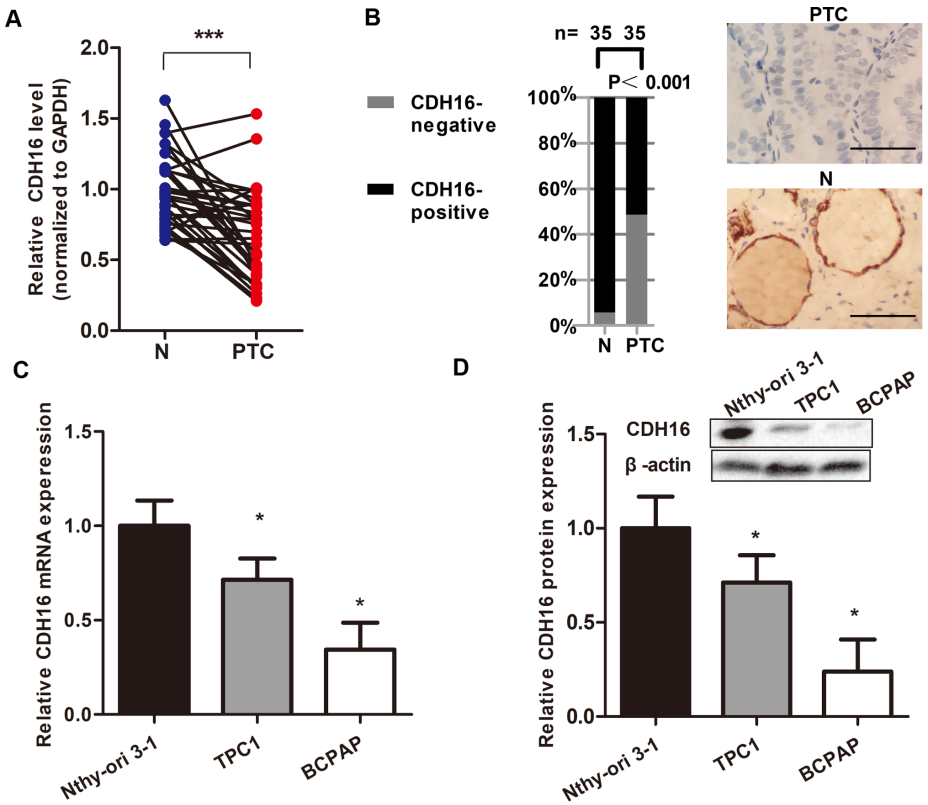

Figure 2. CDH16 expression in 35 PTC tissue samples and PTC and follicular cell lines. Expression levels of CDH16 in 35 pairs of PTC and corresponding adjacent N tissue were evaluated by (A) RT-qPCR and (B) immunohistochemical staining. Scale bar, 25 μm. ***P<0.001 vs. N. CDH16 expression in TC cell lines (BCPAP and TPC1) and the human thyroid follicular cell line Nthy-ori3-1 was detected by (C) RT-qPCR and (D) western blotting. *P<0.05 vs. Nthy-ori3-1 cells. Data are presented as the mean ± SD of three independent experiments. N, normal; PTC, papillary thyroid cancer; CDH, cadherin; RT-q, reverse transcription-quantitative; TC, thyroid carcinoma.

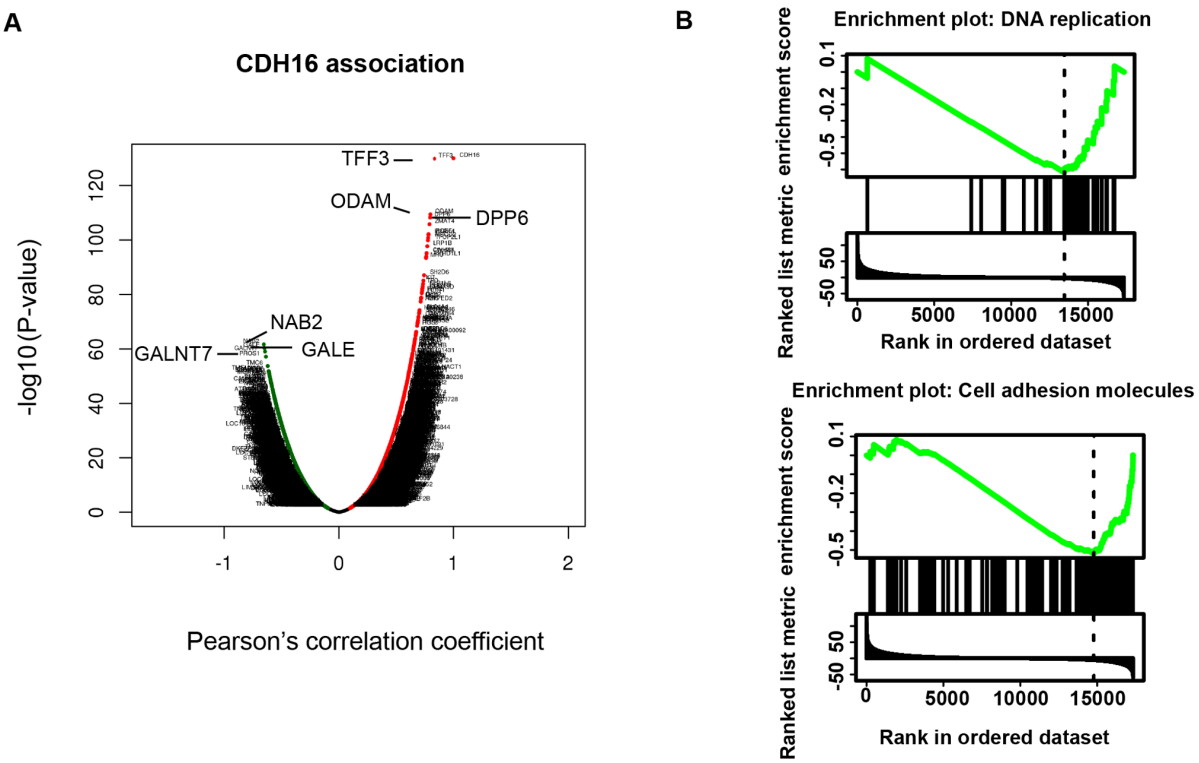

Figure 3. GSEA of co-expressed genes with CDH16 in TC. (A) Volcano map of genes co-expressed with CDH16 in TCGA samples, as evaluated by LinkedOmics. Red, positively correlated; green, negatively correlated. (B) RNA-sequencing data from TCGA were analyzed by GSEA enrichment plots for KEGG_DNA_REPLICATION and KEGG_CELL_ADHENSION_MOLECULES_. KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis; CDH, cadherin.

Table II. Significant enrichment of Kyoto Encyclopedia of Genes and Genomes pathways of cadherin 16 in thyroid carcinoma (LinkedOmics).

Description	Leading edge genes, n	Leading edge gene
DNA replication	24	MCM6; MCM2; MCM7; MCM4; MCM5; POLD1; RFC2; MCM3; LIG1; POLE3; POLE; RFC3; POLE2; PCNA; RNASEHIA; POLD3; POLA2; PRIM1; RFC4; POLA1; FEN1; RNASEH1; RPA2; PRIM2
Cell adhesion molecules	54	CLDN1; SDC4; ICAM1; CDH3; NRCAM; CD276; CLDN16; SDC3; CLDN10; CLDN7; CD58; CLDN4; ITGB8; CDH4; PTPRF; NLGN2; ALCAM; CLDN9; VTCN1; CNTNAP1; F11R; MAG; ITGAM; HLA-8; CDH15; CLDN2; ITGB7; HLA-DQB1; ITGA9; HLA-DRA; HLA-DQA1; HLA-G; CD22; HLA-DOB; HLA-A; HLA-C; HLA-DRB1; ITGB1; HLA-DOA; SELL; SELPLG; CD86; ITGB2; CD274; HLA-DPA1; NCAM2; HLA-DRB5; CD40; PTPRM;ICOSLG; HLA-DQA2; HLA-DPB1; SPN; CLDN3

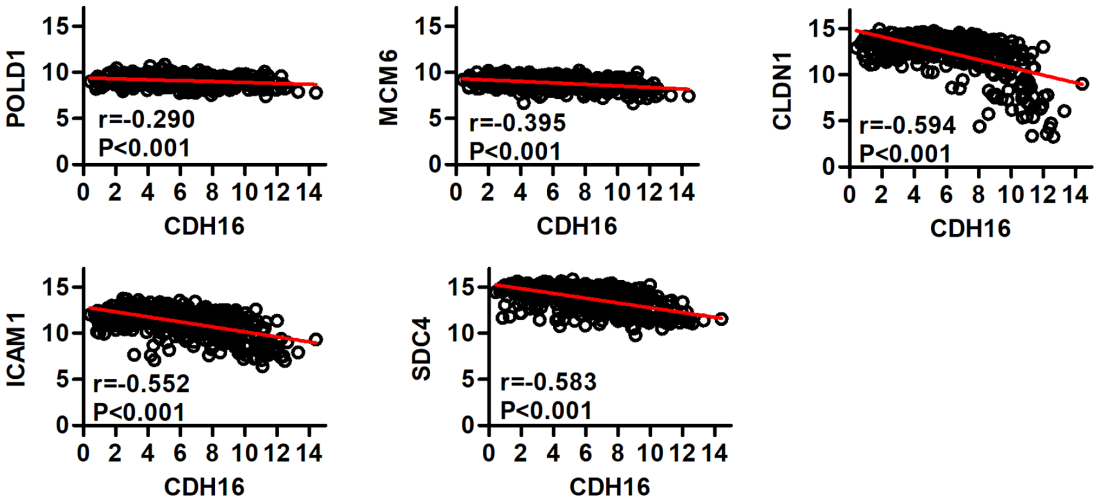

Figure 4. Correlation between POLD1, MCM6, CLDN1, ICAM1, SDC4 and CDH16 expression was analyzed using the LinkedOmics database. POL, polymerase; MCM, minichromosome maintenance; CLDN, claudin; ICAM, intercellular adhesion molecule; SDC, syndecan; CDH, cadherin.

and cell adhesion pathways (Fig. 3B; Table II). Correlation between expression levels of POLD1, MCM6, CLDN1, ICAM1 and SDC4 and those of CDH16 in 503 patients with PTC in TCGA was analyzed. The expression of each of these genes was significantly negatively correlated with expression of CDH16 in PTC (r=-0.290, -0.395, -0.594, -0.552, -0.583, respectively; P<0.001; Fig. 4).

Overexpression of CDH16 inhibits proliferation, migration and invasion of BCPAP cells in vitro. To verify the function of CDH16 in TC cells, BCPAP cells were transfected with CDH16 expression vector. RT-qPCR and western blot assay verified higher mRNA and protein CDH16 levels in CDH16 overexpression compared with negative control BCPAP cells (P<0.05; Fig. 5A and B). To evaluate the effect of CDH16 on proliferation, MTT assay was performed. The proliferation of BCPAP cells was significantly decreased in overexpression compared with negative control cells (P<0.05; Fig. 5C).

To determine whether CDH16 expression affects tumor-associated functions, we the effect of CDH16 overexpression on cell apoptosis, migration and invasion was assessed. Compared with negative control, the CDH16 overexpression group exhibited a significantly higher apoptosis rate, as evaluated by flow cytometry with Annexin V and PI staining (P<0.05; Fig. 5D and E). Cell scratch test indicated that overexpression of CDH16 significantly inhibited migration of BCPAP cells (P<0.05; Fig. 6A and B). Furthermore, Transwell invasion assay indicated that overexpression of CDH16 significantly inhibited invasion capacity of BCPAP cells (P<0.05; Fig. 6C and D). Overall, these results demonstrated that CDH16 promoted apoptosis and inhibited TC cell proliferation, migration and invasion *in vitro*, which is consistent with the pattern of expression in patients with PTC.

Overexpression of CDH16 downregulates POLD1, MCM6, CLDN1, ICAM1 and SDC4 expression in BCPAP cells in vitro. To verify that CDH16 inhibited cell proliferation, migration and invasion via DNA replication and cell adhesion molecular pathways, western blot assay was used to

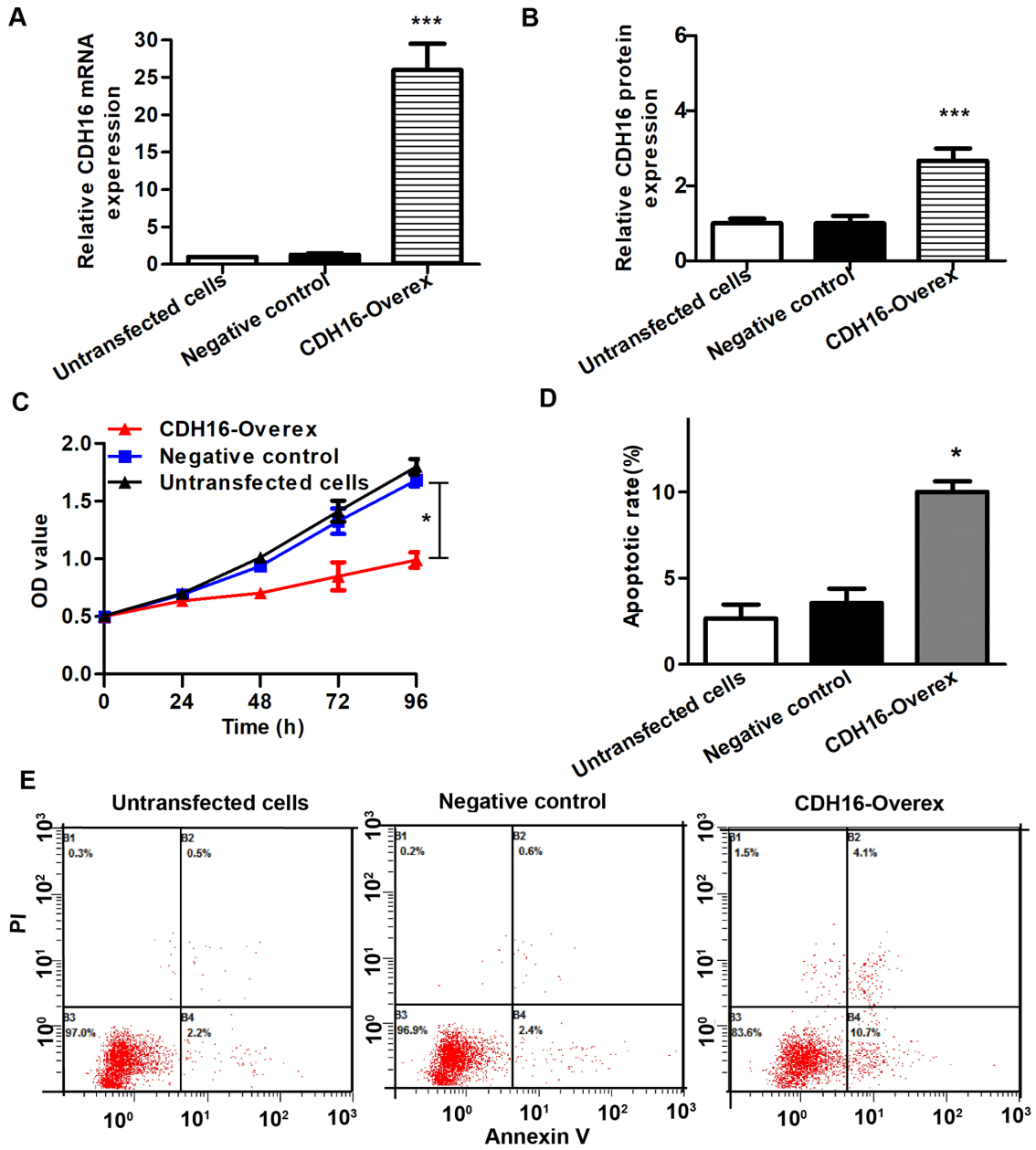

Figure 5. CDH16-Overex inhibits proliferation and promotes apoptosis of BCPAP cells. (A) mRNA levels of CDH16 in CDH16-Overex compared with control cells were examined by reverse transcription-quantitative PCR. (B) Protein levels of CDH16 were assessed by western blotting. (C) Cell proliferation was evaluated by MTT assay and (D) apoptosis was evaluated by (E) flow cytometry following transfection. *P<0.05 and ***P<0.001 vs. negative control. Data are presented as the mean ± SD of three independent experiments. n=3; CDH16, cadherin; Overex, overexpression; OD, optical density.

detect protein expression levels. Following overexpression of CDH16 in TC cells, expression of POLD1, MCM6, CLDN1, ICAM1 and SDC4 was significantly downregulated (53.42±6.38, 37.67±14.27, 42.17±3.36, 37.04±9.34 and 47.83±9.23% decrease, respectively; P<0.05; Fig. 7) compared with negative control. These data indicated that CDH16 may regulate proliferation, migration and invasion of TC cells via DNA replication and cell adhesion pathways.

Discussion

The human genome encodes 115 members of the CDH superfamily and the expression of these family members is tissue-specific (29). CDH1/E-CDH, one of the most classical CDHs, is primarily expressed in epithelial tissue and has been characterized as a tumor suppressor involved in epithelial-mesenchymal transition (30-33). Expression of CDH16 was first observed in the rabbit kidney (34) and has since been shown to be expressed in mouse and human thyroid (17,20). CDH16 co-localizes with CDH1 in thyroid follicular cells and serves a key role in thyroid cell polarity acquisition and follicle formation (18,20). Calì *et al* (20) demonstrated that CDH16 promotes intercellular adhesion to a similar extent as CDH1 and loss of CDH16 precedes loss of CDH1 in TC. CDH1 expression decreases in FTC and has been suggested as a marker for prognosis of TC (35,36). To the best of our knowledge, however, the mechanism of CDH16 in TC has not previously been determined.

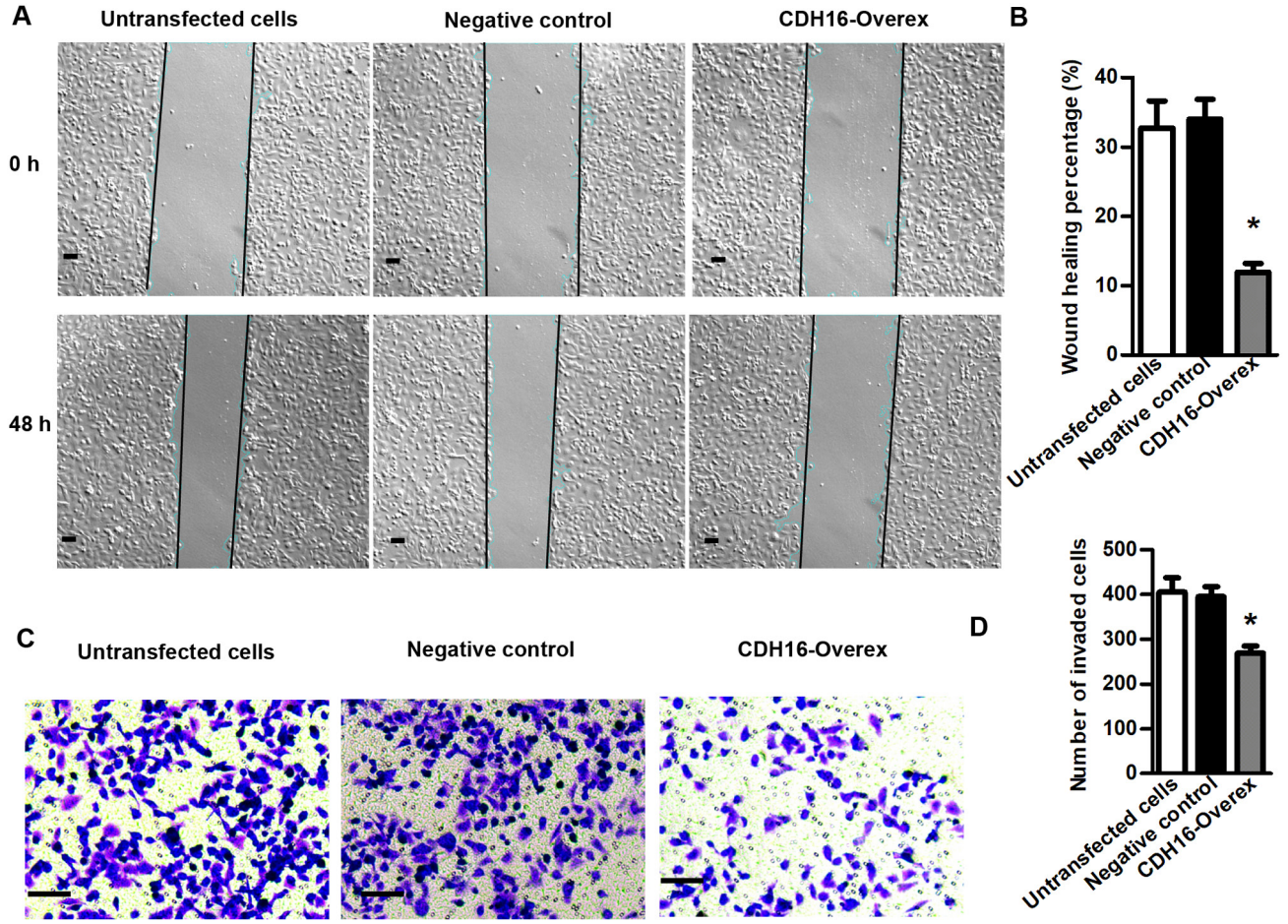

Figure 6. Overexpression of CDH16 inhibits migration and invasion of BCPAP cells. (A and B) Cell migration was evaluated by cell scratch test following CDH16 overexpression. Scale bar, 500 μm. (C) Migration ability of BCPAP cells following transfection was determined by (D) Transwell invasion assay. Scale bar, 100 μm. Data are presented as the mean ± SD of three independent experiments. *P<0.05 vs. negative control. CDH, cadherin; Overex, overexpression.

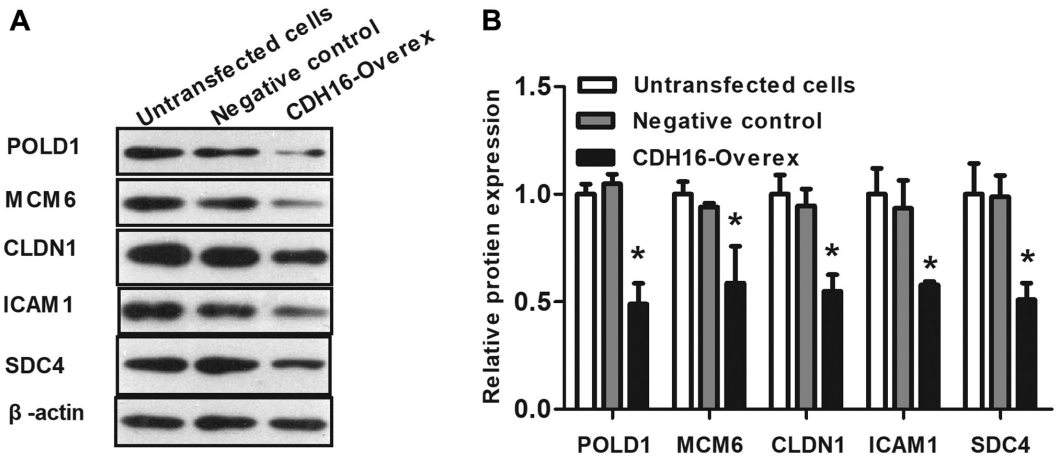

Figure 7. Overexpression of CDH16 downregulates DNA replication and cell adhesion pathway genes. (A) Representative western blots showing downregulation of POLD1, MCM6, CLDN1, ICAM1 and SDC4 following overexpression of CDH16. (B) Relative protein levels were quantified by densitometry of western blots (three replicates). Data are presented as the mean ± SD of three independent experiments. n=3. *P<0.05 vs. negative control. CDH16, cadherin; Overex, overexpression, POL, polymerase; MCM, minichromosome maintenance; CLDN, claudin; ICAM, intercellular adhesion molecule; SDC, syndecan.

The present study characterized CDH16 expression in different forms of TC. The results demonstrated that CDH16 mRNA levels were significantly downregulated in PTC, FTC and ATC compared with normal thyroid tissue. Furthermore, CDH16 ranked within the top 5% of downregulated genes, with the highest rank (<2%) for PTC. These data demonstrated that CDH16 downregulation of expression was a common feature of TC. CDH16 protein expression in TC was correlated with tumor size, disease stage and nodal metastasis in an independent cohort of 35 patients. Consistent with the present results,

Li *et al* (21) demonstrated an association between CDH16 downregulation and lymph node metastasis using TCGA cohorts. These results confirm previous findings suggesting that CDH16 may serve as a biomarker for PTC diagnosis and prognosis (20,21). CNV affects gene expression and is a key pathogenic factor in types of cancer, such as ovarian cancer (37). However, in the present study, no significant CN loss of CDH16 was detected in TC. These results suggested that CDH16 may be a potential diagnostic and prognostic marker for TC and that downregulation of CDH16 was independent of CNV.

CDH16 serves as a tumor suppressor in TC (20,21) but the specific pathogenesis remains largely unclear. Here, CDH16 was co-expressed with genes involved in DNA replication and cell adhesion molecule pathways. To verify the role of CDH16 in TC, CDH16 was overexpressed in BCPAP cells and MTT, flow cytometry, cell scratch and Transwell invasion assays were performed to determine whether overexpression of CDH16 affected proliferation, apoptosis, migration and invasion. The results demonstrated that overexpression of CDH16 resulted in inhibition of proliferation, migration and invasion of TC cells and increased apoptosis.

KEGG analysis demonstrated that CDH16 expression was negatively associated with expression of DNA replication genes. DNA replication is a key event for cell proliferation that is separated into three stages: Initiation, extension and termination (38). At the beginning of replication, the double strands of the DNA helix unwind under the action of helicase (38). Then, using each parent chain as a template and four deoxynucleotides in the surrounding environment as raw materials, a chain complementary to the parent chain is synthesized under the action of DNA polymerase according to complementary base pairing (39). The accuracy of DNA replication in eukaryotes requires proteins such as helicase and DNA POL (40,41). MCM protein complex, which consists of six highly conserved proteins (MCM2-7), initiates DNA replication and unwinding via its replicative helicase activity (42). MCM2-7 proteins are present in proliferating cells (43) and overexpression of MCM2, MCM4 and MCM6 is associated with tumorigenesis (44-46). DNA POL α, δ, and ε are key mediators of DNA replication in eukaryotes (41); their mutation or abnormal expression affects the occurrence, development and invasion of human colorectal cancer, stomach adenocarcinoma and pancreatic adenocarcinoma (47-49). POLD1 encodes DNA POL δ (50); an increase in its protein expression or activity has been demonstrated to be associated with tumorigenesis in colorectal carcinoma and endometrial carcinoma (51-53). In addition, it is associated with the invasive ability of cancer cells. For example, Sanefuji *et al* (54) reported that POLD1 is a key indicator of the activity and invasion of hepatoma cells and its expression is associated with the degree of vascular invasion of cancer cells. Sigurdson *et al* (55) found that increased POLD1 expression increases the risk of breast cancer. The present study showed that expression of MCM6 and POLD1 in TC was significantly negatively correlated with CDH16 by analyzing TCGA thyroid cancer data and that expression of MCM6 and POLD1 was significantly downregulated and cell proliferation inhibited after overexpression of CDH16 in BCPAP cells. This suggested that CDH16 inhibited proliferation of TC cells via downregulation of proteins associated with DNA replication.

KEGG analysis showed that CDH16 was associated with the cell adhesion molecular pathway. Adhesion molecules are divided into five groups: Integrins, selectins, CDHs, hyaladherin and immunoglobulin superfamily members (56). Cell adhesion molecules regulate tumor invasion and metastasis (57). By binding to surface adhesion molecules and extracellular matrix or cell ligands, they activate intracellular signaling pathways and endow tumor cells with metastatic ability (57). CLDNs are cytoskeletal proteins of tight junction between cells that not only regulate paracellular transepithelial/transendothelial transport but are also key for cell proliferation and differentiation (58). The abnormal expression of CLDNs can lead to structural and functional damage of epithelial and endothelial cells, which leads to tumor invasion and metastasis (59). CLDN1 is the most studied CLDN in cancer and its overexpression or loss of expression is observed in different types of cancer (60). CLDN1 expression is low in invasive breast, esophageal and prostate cancer (59-61). However, CLDN1 is highly expressed in ovarian, colon and thyroid cancer (60,62-64). Furthermore, the mechanism of CLDN1 in promoting tumor invasion may involve activation of matrix metalloproteinase 2 (65). Considering the role of CLDN1 in TC, targeting CLDN1 expression may be a promising treatment for TC (66).

Other cell adhesion molecules identified in the present study included ICAM1 and SDC4. ICAM1 is an immune protein superfamily adhesion molecule that is widely expressed on the surface of monocytes, lymphocytes and vascular endothelial and cancer cells (67). Its primary function is to regulate adhesion between cells and between cells and the extracellular matrix. In addition, it is involved in cell signal transduction, proliferation and migration, immune inflammatory response, vascular growth, tumor invasion and metastasis, as well as other physiological and pathological processes (68). ICAM1 is overexpressed in a number of tumor types, including TC (69,70). The binding of ICAM1 and its ligand lymphocyte-associated antigen 1 (LFA1) inhibits natural killer and cytotoxic T cells in the occurrence and development of malignant tumor, helps tumor cells escape immune surveillance and attack and promotes invasion and metastasis of tumor cells (71). SDC, a family of four transmembrane proteoglycans, has been reported to serve key roles in cell proliferation, migration and differentiation (72). SDC4 is highly expressed in TC compared with normal tissue (73). Chen *et al* (73) revealed that SDC4 expression levels are upregulated in PTC and SDC4 gene silencing suppresses PTC cell migration and invasion by suppressing activation of the Wnt/β-catenin signaling pathway.

The present study confirmed that expression of CLDN1, ICAM1 and SDC4 was negatively correlated with CDH16 expression in TCGA databases. Furthermore, expression of CLDN1, ICAM1 and SDC4 was verified by western blotting following overexpression of CDH16. The present results demonstrated that CDH16-overexpressing TC cells exhibited significantly downregulated levels of CLDN1, ICAM1 and SDC4, which lead to inhibition of cell migration and invasion. Thus, CDH16 and cell adhesion pathways may serve a role in TC via migration and invasion, which is consistent with results of KEGG analysis. These results are in accordance with bioinformatics analysis results and support the findings of

the present study, confirming that CDH16 may be a functional tumor suppressor in TC.

In conclusion, the present study used an extended dataset to demonstrate that CDH16 is downregulated in PTC, FTC and ATC and demonstrated that its expression was significantly correlated with tumor size, lymph node metastasis and disease stage in a cohort of 35 patients with PTC. Furthermore, bioinformatics analysis showed that downregulation of CDH16 may promote TC via DNA replication and cell adhesion pathways. The present study also demonstrated that overexpression of CDH16 inhibited proliferation, migration and invasion and induced apoptosis of BCPAP cells. These findings were demonstrated using human tumor samples and cell lines; however, studies in mice would provide *in vivo* verification. To the best of our knowledge, the present study is the first to provide direct evidence for the tumor suppressive effect of CDH16 in TC. While the molecular mechanism of CDH16 disruption in TC remains unknown, elucidating the signaling mechanism underlying CDH16 downregulation in TC may inform the design of novel therapeutic strategies to treat TC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XY, YuL and GL designed the study. XY, WZ and YiL performed the experiments. GL and XY performed data analysis. XY and YuL confirm the authenticity of all the raw data. XY drafted the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Tangshan Gongren Hospital (approval no. GRYY-LL-2020-104). All population-related studies were carried out according to the World Medical Association Declaration of Helsinki. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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