

Original Article

Downregulated CDH3 decreases proliferation, migration, and invasion in thyroid cancer

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Abstract: Background: Placental-Cadherin (CDH3), a cell adhesion molecule, is associated with the function of cells to bind with other cells and the extracellular matrix (ECM). CDH3 is highly expressed in many malignancies, and has been proved it could be a serum marker to monitor colorectal cancer, but the CDH3 expression levels in thyroid cancer is still not clear. In this article, we will illuminate the correlation between CDH3 expression and thyroid cancer. Materials and methods: We analyzed the level of CDH3 expression in 60 pair of tissue samples (contrast thyroid cancer tissues with adjacent normal thyroid tissues) by Real-time PCR, and TCGA data portal. After that, we transfected small interfering RNA to silence CDH3 in thyroid cancer cell lines (KTC-1 and BCPAP) and confirmed the function of CDH3 by performed colony formation, migration, invasion, cell counting kit-8 and apoptosis assays. Results: CDH3 was upregulated in thyroid cancer tissues compared to the adjacent normal tissues (T:N=71.87±39.88:5.35±5.91, P<0.0001) and TCGA (T:N=19.43±13.82:1.22±1.33, P<0.0001). In thyroid cell lines (KTC-1 and BCPAP) experiments showed that downregulated CDH3 inhibited proliferation, migration, and invasion. Meanwhile, inhibited CDH3 expression could upregulate E-cadherin, downregulated N-cadherin, which may control invasion and migration. Conclusion: Thyroid cancer cells CDH3 expression levels is a correlation with its ability to grow, migrate and invade.

Keywords: Thyroid cancer, CDH3, Placental-Cadherin, cell adhesion molecule

Introduction

Thyroid cancer is a common and rapidly increasing malignancy in global, and its incidence continues to climb over the last decade [1, 2]. Its average annual increase incidence was about 6.6%, the highest among all cancer between 2000 and 2009 in USA [2]. It was also exhibited a triple increase over the past decades in many countries [3, 4]. Although the mortality rate of thyroid cancer is relatively low, the recurrence or persistence rate of disease is high, which could be related to increased incurability and patient morbidity and mortality [5]. Exploring the molecular pathogenesis of thyroid cancer could help to find the more-effective treatment strategies. In this study, the focus is on exploring the correlation between CDH3 expression with the malignancy of thyroid tumors.

Classic cadherins are a large superfamily, which includes more than 350 members, in

both vertebrates and invertebrates [6]. They are single-pass transmembrane glycoproteins, consist of the extracellular component that is applied to recognize the similar cadherins in surrounding tissues and cells, and the cytoplasmic domains bind to catenins adjusting cell morphogenesis [7]. They are now known to be involved in many biological processes, such as cell recognition, cell signaling, cell communication, morphogenesis, angiogenesis, and possibly even neurotransmission [8, 9]. The function of control cellular adhesion and bind with other cells and ECM, made it play an important part for cell differentiation, growth, and migration [10, 11]. In a word, cadherins family play important roles in the build and maintenance of tissue form [10-12].

P-cadherin was firstly described in 1986, that appeared in developing mouse embryos [13]. Similarly to other members of the cadherin family, P-cadherin was important for cell growth and migration. But about its carcinogenesis,

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the role of P-cadherin was different in the different tumors [14]. There are studies show that P-cadherin expression in pancreatic cancer, breast cancer, gastric cancer and so on was up-regulation and promoted tumorigenesis [14-17]. However, in some tumors, such as non-small cell lung cancer, hepatocellular carcinoma, and melanoma, the P-cadherin expression was found opposing effects that were down-regulation and suppressed tumorigenesis [14, 18-20]. We found the function of P-cadherin in thyroid cancer is still unclear. In this study, we detected the expression of P-cadherin in thyroid cancer samples compared to adjacent normal tissues by RT-PCT and verified its function in cell lines experiments.

Materials and methods

Patients and thyroid tissue samples

In this study, all samples, 60 pairs of thyroid cancer and adjacent normal tissues, collected from the department of thyroid and breast surgery, at the first affiliated hospital of Wenzhou Medical University.

Major inclusion criteria were: (i) patients with pathologically confirmed thyroid carcinoma in the primary tumor but without any severe diseases in other organs, (ii) patients that had received total/near total thyroidectomy but had not received any radiotherapy, and (iii) patients with a negative history of any other malignant cancers. Major exclusion criteria were: (a) patients with a positive history of other malignant cancers, (b) patients with severe diseases such as heart failure, stroke, and chronic renal failure, and (c) patients with a history of ¹³¹I therapy.

The specimens after surgical resection were immediately transferred to the Liquid nitrogen tank and stored at the -80°C refrigerator. All procedures were approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. The date of CDH3 expression levels of thyroid cancer was download from The Cancer Genome Atlas data portal (TCGA).

Cell culture

The thyroid cancer cell line KTC was purchased from the Chinese Academy of Science (Beijing,

China), and the BCPAP cell line was obtained from Prof. Mingzhao Xing (The Johns Hopkins University, USA). Both cells were incubated at 37°C with 5% CO₂ and nourished by the RPMI 1640 culture medium, containing 10% FBS (Gibco, Grand Island, NY, USA), 100 UI/ml penicillin and 100 µg/ml streptomycin, and stored at 4°C after mixing.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The RNA of cells and tissues were isolated by TRIzol (Invitrogen, USA), experimental procedure strictly adhered to the reagent manufacturer's instructions. Before extracting RNA, the samples stored at -80°C after isolated by TRIzol. Assessed the purity of extracted RNA by spectrophotometry at 260/280 nm. About 1 µg RNA diluted with Nuclease-free water to 14 µl, initial denaturation at 70°C for 10 min, then blend with 4 µl 5XRT Buffer, 1 µl Enzyme Mix, 1 µl Primer Mix, finally there is 20 µl total volume at each tube successively at 16°C for 5 minutes, followed by 95°C for 5 minutes, 42°C for 30 min and held at 4°C. Finally, taking Real-time PCR using SYBR qPCR Mix (Toyobo) according to instructions to analyze the relative expression of CDH3, all data normalized to the GAPDH levels in every sample. The primers sequences in this study shows as follow: CDH3 forward: 5'-TGACCACAAGCCCAAGTTTAC-3' and reverse: 5'-TAAGCAACCACCCATTGTAG-3'; GAPDH: forward: 5'-GTCTCCTCTGACTTCAACAGCG3' and reverse: 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Transient transfection

We use the small interference RNA (siRNA) obtained from GenePharma (Shanghai, China) to silenced CDH3, all cells were distributed in a 6-well plate one day before the deal with siRNA, the process of transient transfection strictly referenced to the product description. The auxiliary transfection reagent lipoiMAX was supplied by Invitrogen. Cells were incubated 48 h after dealing with siRNA, control group cells deal with nontargeting siRNA. The sequences of CDH3 siRNA: CDH3 siRNA-1: Forward 5'-GGAUGUUCCCUAUAUGCAATT-3' and Reverse 5'-UUGCAUAUAGGGAACAUCCTT-3'; CDH3 siRNA-2: Forward 5'-GCCACAUCUGUCAUGUCUUTT-3' and Reverse 5'-AAGACAUGACAGAUGUGGCTT-3'.

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Transwell assay

Transwell assay includes the invasion (Corning, NY, USA) and migration. Transfected cells incubated in 6-well plate were digested by Trypsin-EDTA Solution. Then, the upper chamber of transwell was filled with 3×10^4 for BCPAP or KTC-1 cells resuspended in serum-free medium (300 μ l), while the bottom chamber was filled with RPMI 1640 culture medium (include 10% FBS), then put into incubator about 24 h. During the 24 hours, invasive (or migratory) cells passed through from the upper chamber to the bottom chamber, Before the end, still have a few steps need to complete, fixed with 4% Paraformaldehyde Fix Solution for 15 min, obliterated the exceptional cells with PBS, and at last, stained for 15 min with 0.01% crystal violet solution. The pictures were captured by a photomicroscope.

Colony-forming assay

The cells which had a deal with transient transfection assay (include control group) were digested by Solution. Take 1,500 cells seeded into 6-well plate each well, incubated at 37°C with 5% CO₂. Observed its growth state, we can harvest cells when its cluster includes more than 50 cells probably (about 7-10 days). When the time is right, colonies were fixed with 4% Paraformaldehyde Fix Solution for 15 min, stained for 15 min with 0.01% crystal violet solution, cleaned with pure water before dried. The pictures were captured by the camera. The results were verified by repeated experiments.

CCK-8 proliferation assay

The Cell Counting Kit-8-cell proliferation assay was used to assess the ability of cell growth. Transfected cells seeded into 96-well plates, about 1500 cells/wells and 20 repeats/group, incubated at 37°C with 5% CO₂. Next, every 24 hours, we added 10 μ l CCK-8 reagent to each well with 5 repeats and incubated 2.5 hours, then measured the absorbance at 450 nm. The results were verified by repeated experiments.

Western blot analysis

The protein of transfected cells lysates was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (BioRad, Berkeley, CA, USA) and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA).

Using 5% nonfat milk to block the membranes for 2 h, after that incubated with polyclonal antibody overnight at 4°C, and with the secondary antibody for 1 h at atmospheric temperature, all date normalized to the GAPDH in every sample. The primary antibodies used in this study were as follows: N-cadherin (cat no. 13116; Cell Signaling Technology, Inc., Danvers, MA, USA), E-cadherin (cat no. 3195; Cell Signaling Technology, Inc.), and human β -actin (cat no. 3700; Cell Signaling Technology, Inc.). Then incubated with goat anti-rabbit immunoglobulin G secondary antibody (Alexa Fluor 488; cat. no. ab150077; 1:1,000 dilution; Thermo Fisher Scientific, Inc.) for 2 h in room temperature. The enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) was used to detect the results of western blot analysis. The relative level of each protein was deduced from the ratio of the mean value of each band to that of β -actin.

Statistical analysis

All date of the above experiment is presented as mean \pm SD. In this assay, the software for statistical analysis was GraphPad software version 5.0 (GraphPad Software, CA, USA), Student's t-test was used to distinguish whether there was a significant difference between groups. If $P < 0.05$, there were statistically significant differences between groups.

Results

CDH3 expression is upregulated in thyroid cancer tissues and cell lines

CDH3 is highly expressed in many malignancies, and has been proved it could be a serum marker to monitor colorectal cancer, in our study, we found CDH3 expression is significantly upregulated in thyroid cancer. We used the qRT-PCR to measure the expression levels of CDH3 in 60 pairs of thyroid cancer tissues and paired adjacent normal tissues. The analysis of the data shows that CDH3 is significantly upregulated in thyroid cancers (T:N=71.87 \pm 39.88:5.35 \pm 5.91, paired t-test, $P < 0.0001$) (**Figure 1A**). The result is consistent with the data analysis from TCGA (502 thyroid cancer tissues vs 58 normal tissues, T:N=19.43 \pm 13.82:1.22 \pm 1.33, unpaired t-test, $P < 0.0001$) (**Figure 1B**). To further verify the protein expression of CDH3 in thyroid cancer tissues, we extracted the immunohistochemical staining images from

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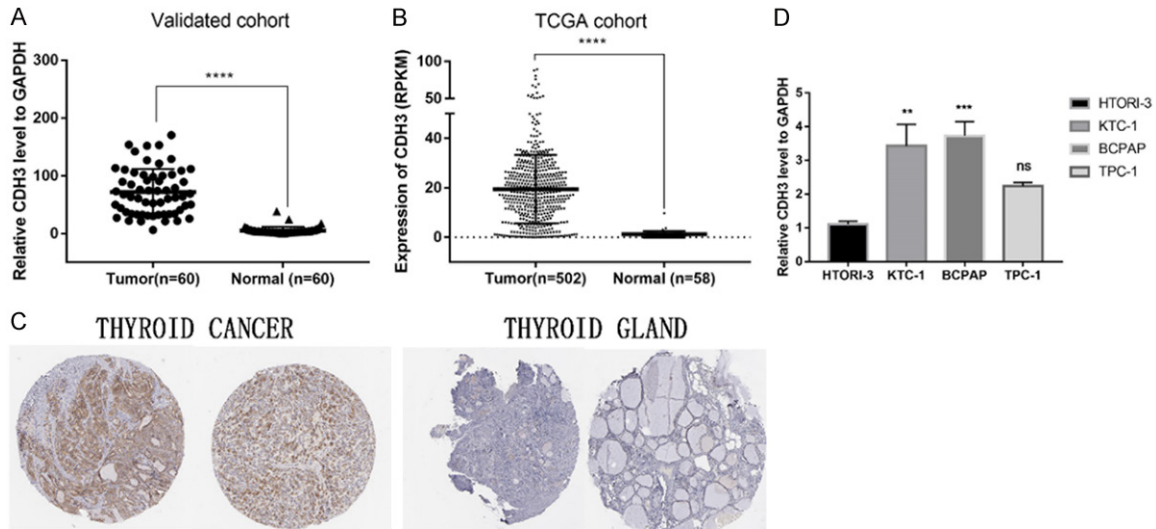


Figure 1. CDH3 expression in thyroid cancer in the validated cohort, TCGA cohort, and thyroid cancer cell lines. A. The relative CDH3 expression was examined by RT-PCR in 60 pairs of thyroid cancer samples and adjacent normal tissues (paired t-test, $P < 0.0001$). B. The relative CDH3 expression in TCGA contained 502 thyroid cancer samples and 58 normal tissues (unpaired t-test, $P < 0.0001$). C. The relative CDH3 expression in thyroid cancer cells (compared to HTORI-3), KTC-1 and BCPAP exhibited a higher expression compared to TPC. D. The expression of CDH3 performs a higher level compared with normal cells line (compared to HTORI-3, KTC-1, $P < 0.01$; BCPAP, $P < 0.001$) $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Table 1. The relationship between CDH3 and clinicopathologic characteristics in the TCGA cohort

Clinicopathologic characteristics	Low expression (%)	High expression (%)	χ^2	P-value
Age (years)			3.97	0.046*
<45	101	125		
≥ 45	148	128		
Gender			0.66	0.416
Male	71	64		
Female	178	189		
Tumor size (cm)			0.05	0.832
≤ 2	72	71		
> 2	176	181		
Lymph node metastasis			16.5	$< 0.001^*$
No	131	98		
Yes	85	138		
Clinical stage			1.22	0.269
I-II	171	162		
III-IV	77	90		

Note: * P -value $< .05$.

the Human Protein Atlas database. We found that CDH3 expression in thyroid cancer tissues was increased compared with normal thyroid tissues (**Figure 1C**). It suggests that upregulation is associated with the occurrence, development of cancer. In a variety of thyroid cancer cell lines, the expression of CDH3 also performs a higher level compared with normal cells line (compared to HTORI-3, KTC-1, $P < 0.01$; BCPAP, $P < 0.001$) (**Figure 1D**).

CDH3 expression is associated with clinical features

Analyzed the clinical feature of the 502 thyroid cancer tissues from TCGA, patients were the group by median value into Low-expression and High-expression. We found the age ($P = 0.045 < 0.05$) and the lymph node metastasis ($P < 0.001$) were significant differences in different expression levels groups (**Table 1**). In our validated

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Table 2. The relationship between CDH3 and clinicopathologic characteristics in the validated cohort

Clinicopathologic characteristics	Low expression (%)	High expression (%)	χ^2	P-value
Age (years)			0.205	0.651
<45	13	13		
≥45	19	15		
Gender			0.402	0.526
Male	14	10		
Female	18	18		
Tumor size (cm)			4.25	0.039*
≤1	15	6		
>1	17	22		
Lymph node metastasis			0.402	0.526
No	14	10		
Yes	18	18		
Clinical stage			0.021	0.886
I-II	20	18		
III-IV	12	10		

Note: *P-value <.05.

Table 3. Univariate logistic regression analysis for the lymph node metastatic risk

Clinicopathologic features	OR	95% CI	P-value
CDH3 expression	2.17	1.490-3.162	<0.001
Age	0.608	0.418-0.883	0.009
Gender	0.645	0.425-0.978	0.039
Tumor size	2.525	1.652-3.858	<0.001
Clinical stage	3.493	2.316-5.268	<0.001

Table 4. Multivariate logistic regression analysis for the lymph node metastatic risk

Clinicopathologic features	OR	95% CI	P-value
CDH3 expression	1.991	1.286-3.081	0.002
Age	0.01	0.001-0.072	<0.001
Clinical stage	169.998	22.729-1271.475	<0.001

cohort, we found that the higher expression level of CDH3 had a larger tumor size (P=0.002) compared to the lower expression group (**Table 2**). Univariate logistic regression analysis indicated that the significantly variables for lymph node metastasis were CDH3 expression (odds ratio [OR] 2.17, 95% CI 1.490-3.162, P<0.001), age (OR 0.608, 95% CI 0.418-0.883, P=0.0009), gender (OR 0.645, 95% CI 0.425-0.978, P=0.039), tumor size (OR 2.525, 95% CI 1.652-3.858, P<0.001), and clinical stage (OR 3.493, 95% CI 2.316-5.268, P<0.001) (**Table 3**). In **Table 4**, multivariate logistic analysis using the three mentioned parameters also

revealed that CDH3 expression (odds ratio [OR] 1.991, 95% CI 1.286-3.081, P=0.002) and clinical stage (OR 169.998, 95% CI 22.729-1271.475, P<0.001) were positively correlated with more lymph node metastasis, whereas age (OR 0.01, 95% CI 0.001-0.072, P<0.001).

The expression levels of CDH3 and the risk of lymph node metastasis in thyroid cancer was a positive correlation

There is a significant difference in lymph node metastasis between the high CDH3 expression group and Low CDH3 expression group in TCGA thyroid cancer tissues. To validate the correlation of CDH3 expression with lymph node metastasis, we analyzed by logistic regression. Univariate logistic regression analysis indicated that the significant variables for lymph node metastasis were CDH3 expression (odds ratio [OR] 2.17, 95% CI 1.49-3.162, P<0.001), age (OR 0.698, 95% CI 0.418-0.883, P=0.009<0.01), gender (OR 0.645, 95% CI 0.425-0.978, P=0.039<0.05), tumor size (OR 2.525, 95% CI 1.652-3.858, P<0.001), and clinical stage (OR 3.493, 95% CI 2.316-5.268, P<0.001) (**Table 3**). Multivariate logistic analysis revealed that CDH3 expression (OR 1.991, 95% CI 1.286-3.081, P=0.002<0.01), age (OR 0.01, 95% CI 0.001-0.072, P<0.001) and clinical stage (OR 169.998, 95% CI 22.729-1271.475 P<0.001) were associated with lymph node metastasis (**Table 4**). (OR>1 positive correlation, OR<1 negative correlation). All

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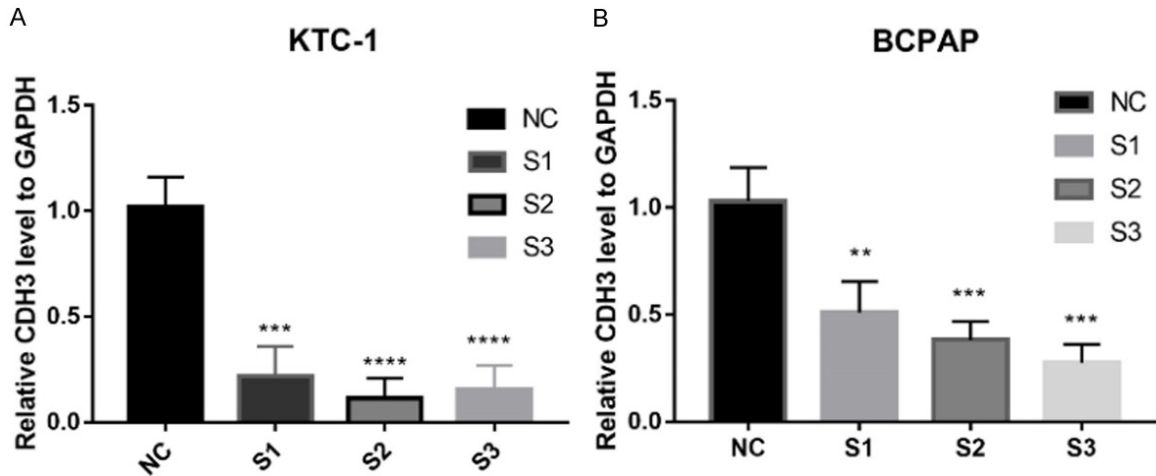


Figure 2. CDH3 expression in thyroid cancer cell lines after transfected with siRNA by RT-qPCR. A. CDH3 expression in KTC-1 after transfected with siRNA. (**P<0.01, ****P<0.0001). B. CDH3 expression in BCPAP after transfected with siRNA. (**P<0.01, ***P<0.001).

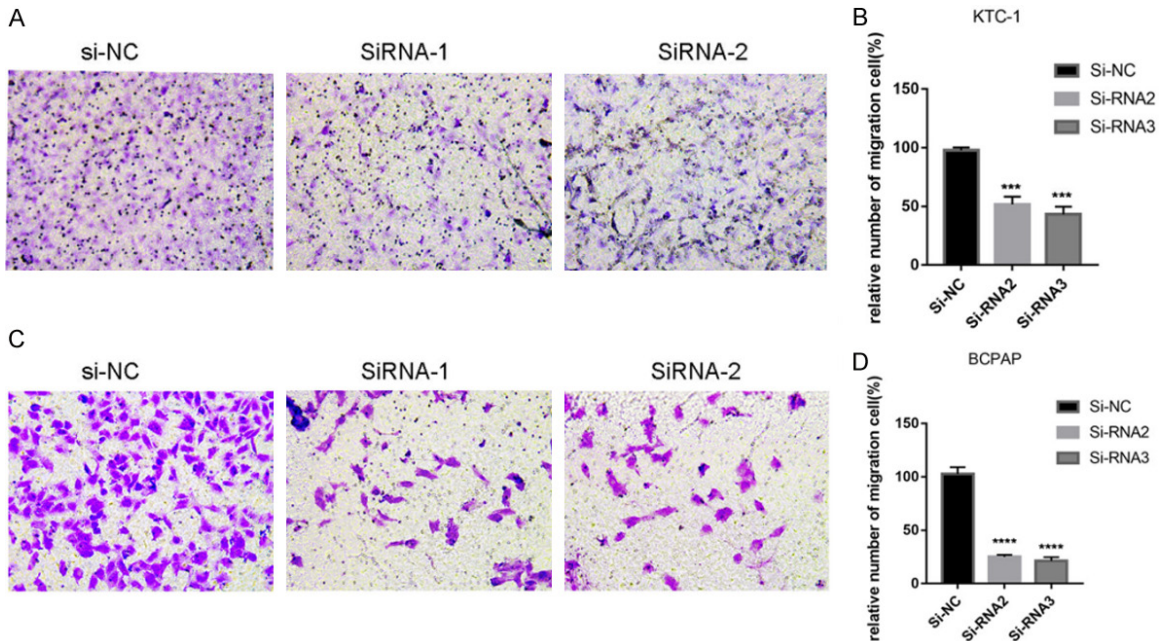


Figure 3. Down-regulation CDH3 expression in KTC-1 and BCPAP cells inhibiting migration. (A) Transwell migration assays in knock-down of CDH3 cells compared with corresponding control cells, in KTC-1 cells. (B) The histogram analysis of (A), Error bars represent SD of the mean, ***P<0.001, Student's t-test. (C) Transwell migration assays in knock-down of CDH3 cells compared with corresponding control cells, in BCPAP cells. (D) The histogram analysis of (C), Error bars represent SD of the mean, ****P<0.0001, Student's t-test.

the above suggested the expression levels of CDH3 and the risk of lymph node metastasis in thyroid cancer was a positive correlation.

The downregulation of CDH3 suppressed the ability of migration and invasion

Invasion and migration assay often use to evaluate tumor metastasis capacity. We found that

downregulating the expression level of CDH3 suppresses the cell line migrate from the upper chamber to the bottom chamber, and there are differences with the NC group under the microscope (**Figure 3**). The same phenomenon happened in the invasion assay (**Figure 4**). In a word, there is a positive correlation between the CDH3 expression and thyroid cancer metastasis capacity.

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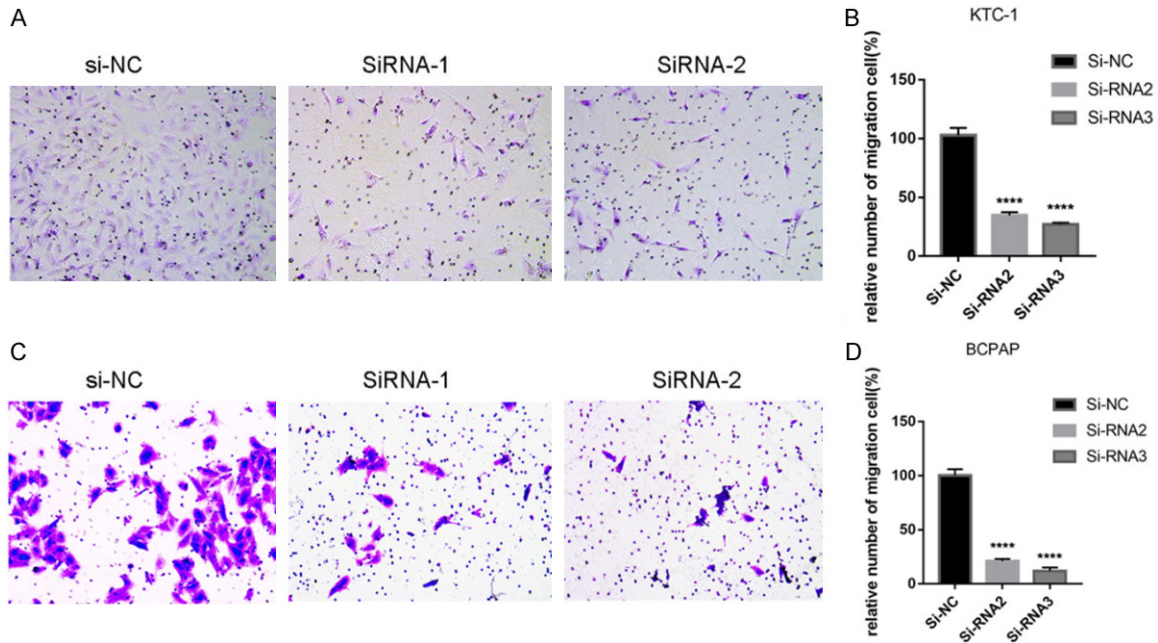


Figure 4. Down-regulation CDH3 expression in KTC-1 and BCPAP cells inhibiting invasion. (A) Transwell invasion assays in knock-out of CDH3 cells compared with corresponding control cells, in KTC-1 cells. (B) The histogram analysis of (A), Error bars represent SD of the mean, **** $P < 0.0001$, Student's t-test. (C) Transwell invasion assays in knock-out of CDH3 cells compared with corresponding control cells, in BCPAP cells. (D) The histogram analysis of (C), Error bars represent SD of the mean, **** $P < 0.0001$, Student's t-test.

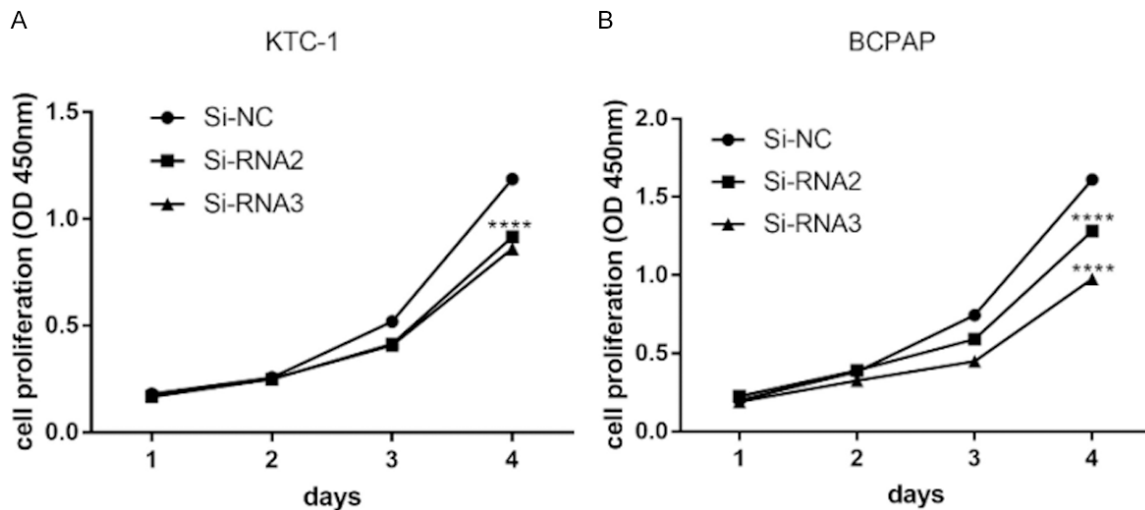


Figure 5. Down-regulation CDH3 expression in KTC-1 and BCPAP cells inhibited proliferation. A. Cell proliferation assay in knock-out of CDH3 cells compared with corresponding control cells, in KTC-1 cells, cell proliferation was measured using CCK-8. **** $P < 0.0001$. B. Cell proliferation assay in knock-out of CDH3 cells compared with corresponding control cells, in BCPAP cells, cell proliferation was measured using CCK-8. **** $P < 0.0001$.

Low expression of CDH3 could inhibit proliferation in thyroid cancer

We use the small interference RNA (siRNA) to silenced CDH3 (Figure 2). To compare with the

control group, three groups of Si all succeeded in silenced. In the following experiments, we choose two of siRNAs to complete. We assess the ability of cell growth by CCK-8 proliferation assay (Figure 5) and Colony-forming assay

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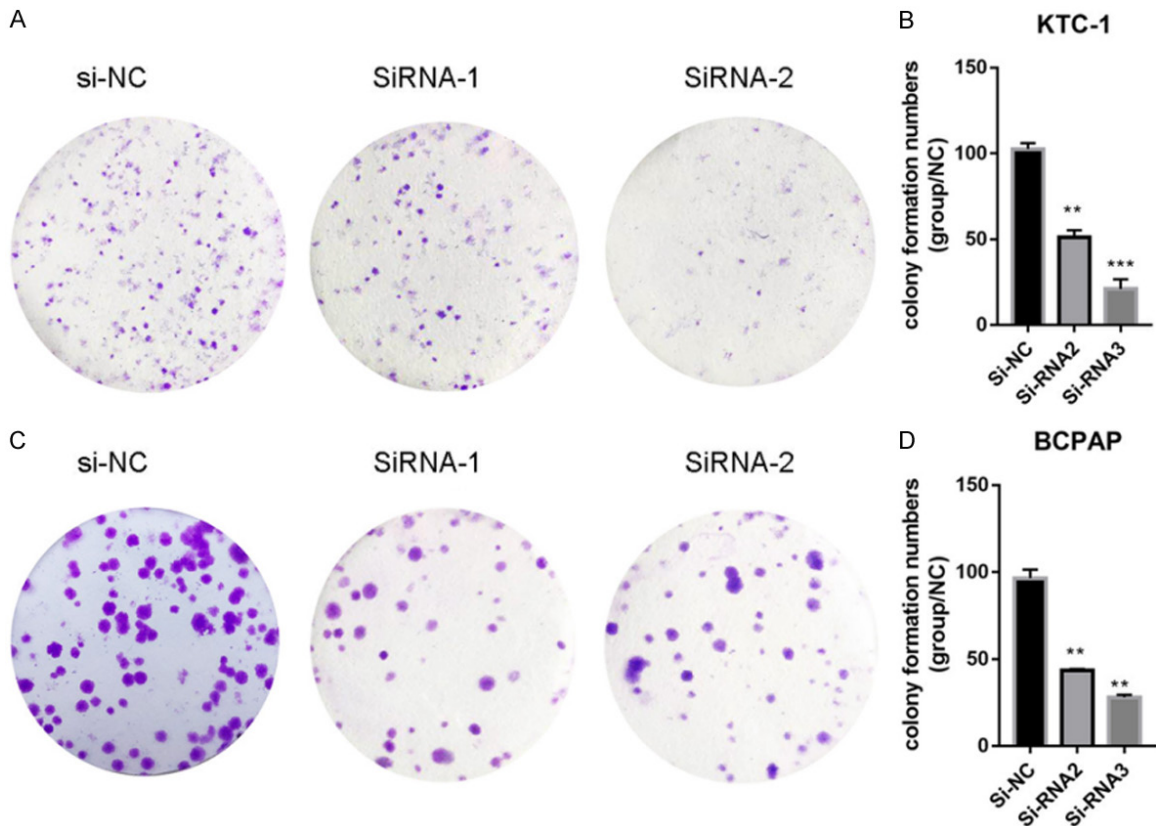


Figure 6. Down-regulation CDH3 expression in KTC-1 and BCPAP cells inhibited colony formation. (A) Colony formation assays in knock-out of CDH3 cells compared with corresponding control cells, in KTC-1 cells. (B) The histogram analysis of (A), Error bars represent SD of the mean, **P<0.01, ***P<0.001, Student's t-test. (C) Colony formation assays in knock-out of CDH3 cells compared with corresponding control cells, in BCPAP cells. (D) The histogram analysis of (C), Error bars represent SD of the mean, **P<0.01, Student's t-test.

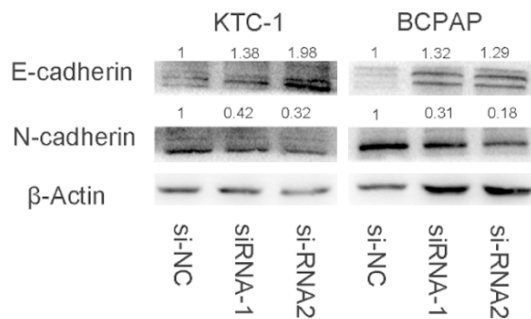


Figure 7. Dysregulation CDH3 regulates KTC-1 and BCPAP cells migration and invasion via EMT. The influence of CDH3 expression on the levels of E-cadherin and N-cadherin in KTC-1, BCPAP cell lines by western blot.

(Figure 6), we found that downregulating the level of CDH3 expression significantly inhibits proliferation in thyroid cancer cell line (KTC and BCPAP).

CDH3 promoted thyroid cancer migration and invasion by regulating EMT

Epithelial-mesenchymal transition (EMT) has emerged as a key regulator of metastasis in some cancers by conferring an invasive phenotype [37]. We have found CDH3 facilitating invasion and migration, so we verify its correlation with EMT by Western blot. We find knockdown CDH3 increased E-cadherin expression and decrease N-cadherin (Figure 7). So, we can find CDH3 promoted thyroid cancer migration and invasion by regulating EMT.

Discussion

With the development of economic and the improvement of living standards, people become more and more concerned about health. With the popularization of executive physical examination and the deeper recognition of the thyroid carcinoma, the occurrence rate of the

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thyroid cancer tends to ascend in recent years. The technology of fine-needle aspiration cytology (FNAC) is the most reliable process to biopsy thyroid nodules, but they still yield indeterminate results about 10% to 40% of all samples [21-23]. Meanwhile, the thyroid microcarcinoma still has a high risk of false negatives on diagnosis by sole FNAC [23, 24]. With the development and application of genomics, detecting puncture specimen in gene levels can help improve the accuracy of diagnosis [25-27]. To find the biomarkers that can distinguish between malignant tumors and benign nodules is needed.

As we all know, *BRAF* V600E is the most common oncogenic mutation in thyroid cancer [28], and highly specific for papillary thyroid carcinoma (PTC). The test of *BRAF* V600E mutation can effectively increase the diagnostic accuracy of FNAC [29, 30]. It is almost 100% specificity for PTC that make potentially accurate markers for diagnostic the indeterminate thyroid in histology by FNAC [30-32].

The cell adhesion molecule expression has been proved to be associated with tumor progression [10, 11]. In previous studies, we found P-cadherin overexpression in breast cancer and colorectal cancer, and it has potential as a serum marker for monitoring [15, 16, 33]. In our current study, we found there is a high expression level of P-cadherin in thyroid cancer tissues and verified in TCGA. We analyzed the clinical dates from the TCGA thyroid cancer patients, found that age ($P=0.001$) and lymph node metastasis ($P=0.002$) were significantly related to the *CDH3* expression. All the above show that *CDH3* may play an important role in thyroid cancer. So we made the function assays in cell lines (BCPAP, KTC) and found downregulated *CDH3* suppressed the ability of migration and invasion and inhibited proliferation.

In recent decades, there is some studies suggest that during cancer progression, cancer cells acquire several hallmarks that promote tumor growth and invasion. A crucial mechanism by which carcinoma cells enhance their invasive capacity is the dissolution of intercellular adhesions and the acquisition of a more motile mesenchymal phenotype as part of an epithelial-to-mesenchymal transition (EMT) [34-36]. So we tested the EMT-related epithelial marker and found that inhibiting *CDH3*

expression could upregulated E-cadherin and downregulated N-cadherin in thyroid cancer cells. All the above mean that decreasing *CDH3* expression could inhibit the epithelial-to-mesenchymal transition to suppress thyroid cancer cell line migration and invasion.

However, there is still an unknown about *CDH3*'s mechanism of action. Further study is needed to explore the via, verify these results and determine their significance.

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Written informed consent was obtained from each individual participant.

Disclosure of conflict of interest

None.

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