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Original Article

Contactin 1 as a potential biomarker promotes cell proliferation and invasion in thyroid cancer

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Abstract: Contactin 1 (CNTN1) as a member of the immunoglobulin superfamily plays important role in the development of nervous system. Recent studies find that elevated CNTN1 can promote the metastasis of cancer. However, the expression and function of CNTN1 in thyroid cancer are still unknown. Here, we firstly find CNTN1 is a new gene which can be regulated by RET/PTC3 (Ret proto-oncogene and Ret-activating protein ELE1) rearrangement gene and the protein level of CNTN1 is increasing in thyroid cancer. Besides this change is positively associated with the TNM stage and tumor size. Moreover, we confirm that knockdown of CNTN1 significantly inhibits the tumor proliferation, invasiveness and represses the expression of cyclin D1 (CCND1). In conclusion, CNTN1 will be a poteintial diagnosis biomarker and therapy target for thyroid cancer.

Keywords: Thyroid cancer, RET, rearrangement, contactin 1, biomarker, metastasis

Introduction

Thyroid cancer (TC) is the most prevalent endocrine cancer and one of the fastest growing diagnoses worldwide, however the cause and mechanism of it are still poorly understood [1]. Receptor tyrosine-protein kinase RET is involved in numerous process of development. Gene rearrangement of RET/PTC3 (Ret proto-oncogene and Ret-activating protein ELE1) is associated with the development of various types of cancer, including multiple endocrine neoplasia, Hirschsprung disease, and medullary thyroid carcinoma [2-4]. Many studies have demonstrated BRAF or RAS mutations, together with RET/PTC3 rearrangement may be the origin of thyroid cancer especially papillary thyroid cancer [5, 6]. And most functions of RET are mediated through the pathways such as ERK, JNK, and PI3k/AKT [7, 8].

CNTN1 is a glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein that functions as a cell adhesion molecule [9]. It promotes oligodendrocyte maturation and myelination by acting as a functional ligand for Notch [10-12]. Absence of CNTN1 can result in deficits in inhibitory synaptic development [13]. Recent study finds that CNTN1 could promote the metastasis of lung cancer by reducing E-cadherin expression [14]. Liu et al demonstrate overexpression of CNTN1 in oesophageal squamous cell carcinomas is correlated with advanced clinical stage and lymph node metastasis [15]. These results are also validated in oral squamous cell carcinoma [16]. However, the mechanism for the regulation of CNTN1 is still blur.

In the present work, we perform Bio-information analysis to seek some new mechanism of thyroid cancer development. Fortunately, we find that CNTN1 may be a novel downstream molecular of RET/PCT3 rearrangement gene which plays key role in the origin of thyroid cancer. Then we detect the protein level of CNTN1 in a series of 100 patients with thyroid cancer by immunohistochemistry, and investigate its relation with clinicopathologic factors. Moreover, the function of CNTN1 in thyroid cancer is confirmed by cell proliferation assay, Migration and invasion assay. And the expression of NOTCH1 target gene (CCND1) is detected by Western Blot assay.

Materials and methods

Patient's samples

100 pairs of formalin-fixed paraffin embedded specimens used for immunohistochemistry

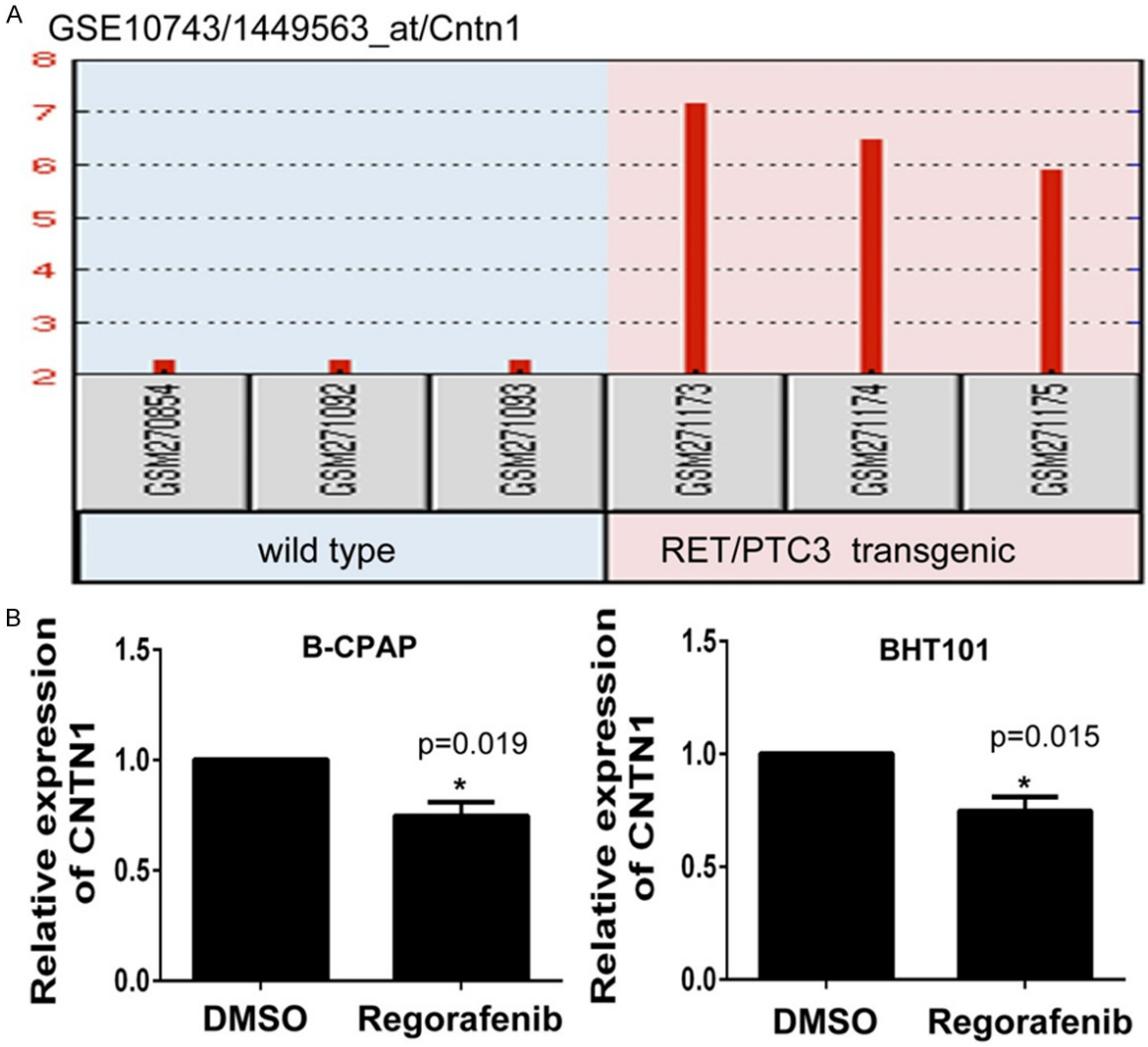

Figure 1. CNTN1 can be regulated by RET/PTC3 rearrangement gene A. Analysis of RET/PTC3 downstream gene in GEO database. B. The expression of CNTN1 was detected by Real time PCR after blocking RET by regorafenib.

were collected from patients undergoing surgical resections in the Zhejiang Cancer Hospital from 2013 to 2014. All the cancer and paracancer Specimens (tissues with normal glandular structure) were based on pathological evidence. And no patients received preoperative chemotherapy or radiotherapy. All the samples were obtained with informed consent and the project was approved by Medical Ethics and Human Clinical Trial Committee.

Bio-information analysis

The target gene of RET/PTC3 and the expression of CNTN1 were analyzed by GEO database software (GSE10743). Protein interaction is analyzed by String.10 software (http://www.string-db.org/).

Immunohistochemical staining

Immunohistochemical staining was performed using Elivision™ plus Kit, and detected by DAB kit (Maixin, China). The slides were incubated over 4 h at 65°C. Antigen retrieval was performed using citrate buffer (pH 6.0) and sections were held in phosphate buffered saline (PBS). After blocking with goat serum, the slides were incubated with monoclonal CNTN1 antibody (1:50, Santa Cruz Biotechnology) at 4°C overnight. Then the sides were incubated with Elivision™ plus polyer HRP (Maixin, China), and the protein was detected by DAB system kit. Cells had brown granules were taken as positive stained. And all the immunoreactions were separately evaluated for positive DAB staining by two independent pathologists. Slides incu-

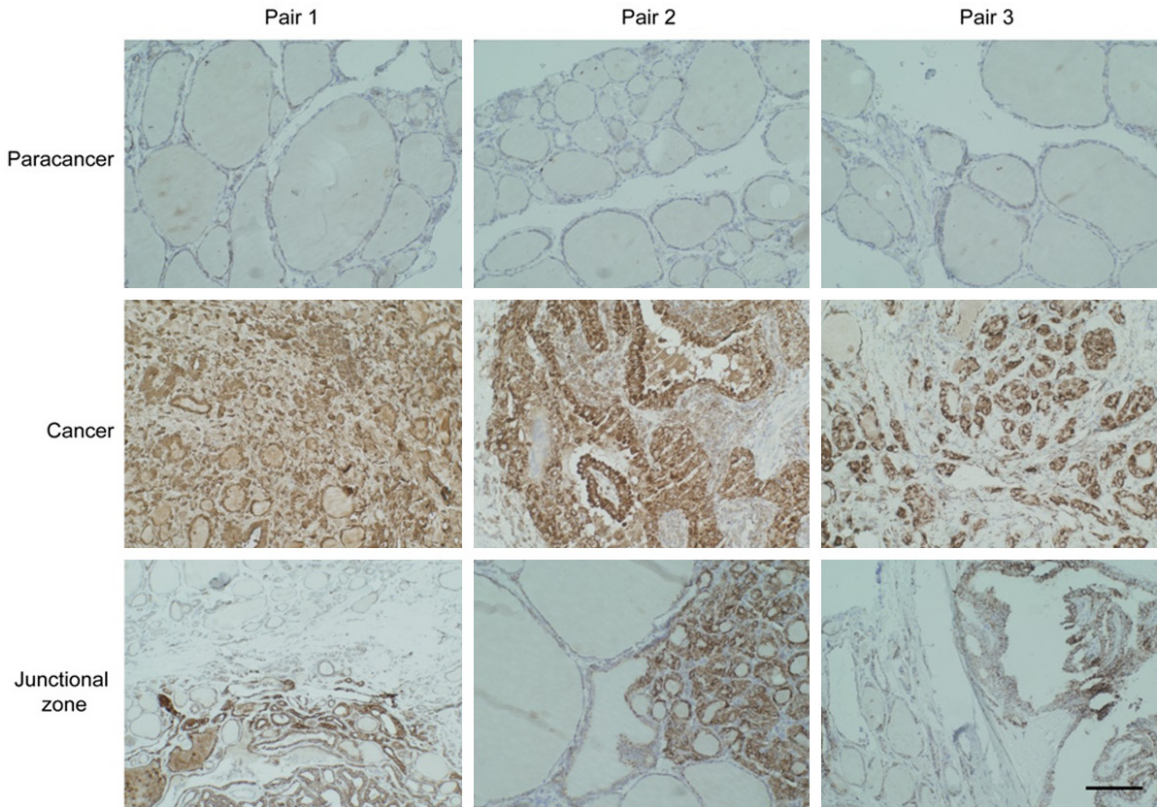

Figure 2. Immunohistochemical staining for CNTN1 in 100 pairs of thyroid cancer. The formalin-fixed paraffin embedded paracancer and cancer specimens which had been confirmed by pathobiology were used. The expression of CNTN1 in paracancer, cancer and junctional zone were stained and photographed, magnification, × 100, and the scale bar size is 100 μm.

Table 1. Comparison between protein expression of CNTN1 in paracancer and cancer tissues

Type	NO	Positive N (%)	*P* value[a]
Paracancer	100	10 (10%)	< 0.001*
Cancer	100	81 (81%)	

[a]Chi square test was used for analysis, *stands for significant difference at $P < 0.05$.

bated with PBS instead of CNTN1 antibody were selected as negative control.

Cell culture and transfection

Thyroid cancer cell lines B-CPAP and BHT101 were purchased from Cell Bank of Chinese Academy of Medical Science (Shanghai, China). Cells were cultured in DMEM or RPMI-1640 medium containing 10% fetal bovine serum (Life technologies, USA), 100 units/ml penicillin and 100 units/ml streptomycin at 37°C in 5% incubator.

For transfection, cells were transfected with 100 nm siRNA which was consisted of pools of three to five target-specific 19-25 nt siRNAs designed to CNTN1 (Santa Cruz Biotechnology, USA) or 100 nm control RNA using Lipofectamine 2000 (Life technologies, USA) following the manufacturer's instructions. The mRNA or protein were harvested and detected after 60 h.

Cell proliferation assay

Cells were digested and seeded into 96-well plates after transfection with CNTN1 siRNA or control RNA. Then cells were incubated for 4 h in the presence of 20 μl MTT solution (5 mg/ml, Sigma), discarded the supernatant and added 200 μl DMSO, Finally, the spectrophotometric absorbance at 490 nm was measured with enzyme-labeling instrument.

Western blot analysis

Cell total protein was extracted with RIPA lysis Buffer (Thermo Fisher Scientific, USA) contain-

Table 2. Correlation of CNTN1 expression with clinical characteristics in Thyroid Cancer

Characteristics	Negative (N = 19) n (%)	Positive (N = 81) n (%)	*P* value[a]
Age			
< 45	8 (42.1%)	35 (43.2%)	0.930
≥ 45	11 (57.9%)	46 (56.8%)	
Gender			
Male	13 (68.4%)	50 (61.7%)	0.587
Female	6 (31.6%)	31 (38.3%)	
Tumor size			
≤ 2 cm (T1)	14 (73.7%)	29 (35.8%)	0.003*
> 2 cm (T2-T4)	5 (26.3%)	52 (64.2%)	
LN status			
Negative (N0)	12 (63.2%)	60 (74.1%)	0.340
Positive (N1)	7 (36.8%)	21 (25.9%)	
TNM stage			
I, II	16 (84.2%)	47 (58%)	0.033*
III, IV	3 (15.8%)	34 (42%)	

[a]Chi square test was used for analysis, *stands for significant difference at $P < 0.05$.

ing protease inhibitor (Roche, USA). Then protein was separated by 8% SDS-PAGE, and transferred to PVDF membrane (Amersham, USA). The membrane were blocked with 5% defated milk, and incubated with CNTN1 antibody (1:500, Santa Cruz Biotechnology), GAPDH antibody (1:5000, Kangchen Inc) overnight. Finally, the membrane was probed with HRP (horseradish peroxidase) labeled goat-antimouse IgG (1:5000, Zhong Shan Jin Qiao Inc) and detected by chemiluminescence.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from cultured cells using Trizol Reagent (Invitrogen). cDNA was synthesized using the PrimeScript™ RT Reagent Kit (Takara, China). Then the cDNA was amplified with SYBR® premix ExTaq™ II (Takara, China) with the following primers: CNTN1-forward, 5'-CAGCCCTTTCCCGGTTTACAA-3', CNTN1-reverse, 5'-TGCTTCTGACCATCCCGTAGT-3'; GAPDH-forward, 5'-CTGGGCTACACTGAGCACC-3', GAPDH-reverse, 5'-AAGTGGTCGTTGAGGGCAATG-3'. Levels of gene expression were determined by ΔΔCT method with the results being expressed as mRNA expression levels normalized to the level of GAPDH.

Migration and invasion assay

For migration assay, 1×10^5 cells (100 ul serum free medium) transfected with CNTN1 RNAi or control RNA were seeded on the upper chamber of the transwell room, and 500 μl complete medium (containing 10% FBS, 100 units/ml penicillin and 100 units/ml streptomycin) were added to the lower chamber overnight. Cells in the upper of the insert were removed by wiping with a cotton swab, fixed and stained with 4% crystal violet. Cell numbers at the bottom of the insert were counted in five random fields. For invasion assay, the upper chamber should be coated with 100 ul matrigel solution (BD Biosciences, USA) before cells seeding. Then the experiment was performed following the migration assay procedure.

Statistical analysis

The statistical significance of differences between groups was assessed using SPSS 16.0 and Graphpad Prism 5. Data were expressed as mean ± SD, χ^2 test was used to examine possible correlations between CNTN1 expression and clinical characteristics. For all the tests, differences with $P < 0.05$ were considered statistically significant.

Results

CNTN1 was a downstream protein of RET/PTC3 fusion gene

RET/PTC3 rearrangements was the most frequent genetic alterations in thyroid cancer [17], so we firstly analyzed the potential downstream gene of RET/PTC3 in GEO database. As shown in **Figure 1A**, the expression of CNTN1 was significantly raised in RET/PTC3 transgenic mouse. This result suggested that CNTN1 was involved in the RET/PTC3 pathway. To further confirm this result, cells were treated with a RET inhibitor regorafenib to block the activity of RET. From the result we could find that the expression of CNTN1 decreased (**Figure 1B**).

CNTN1 was up-regulated in thyroid cancer

Next, we sought to assess the expression of CNTN1 in thyroid cancer. 100 pairs of thyroid cancer tissues were collected and analyzed by Immunohistochemical staining Assay. Fortunately, we found the expression of CNTN1 was obviously elevated in the tumor tissues by comparing with its expression in paracancer tissues. This change became clearer in the junc-

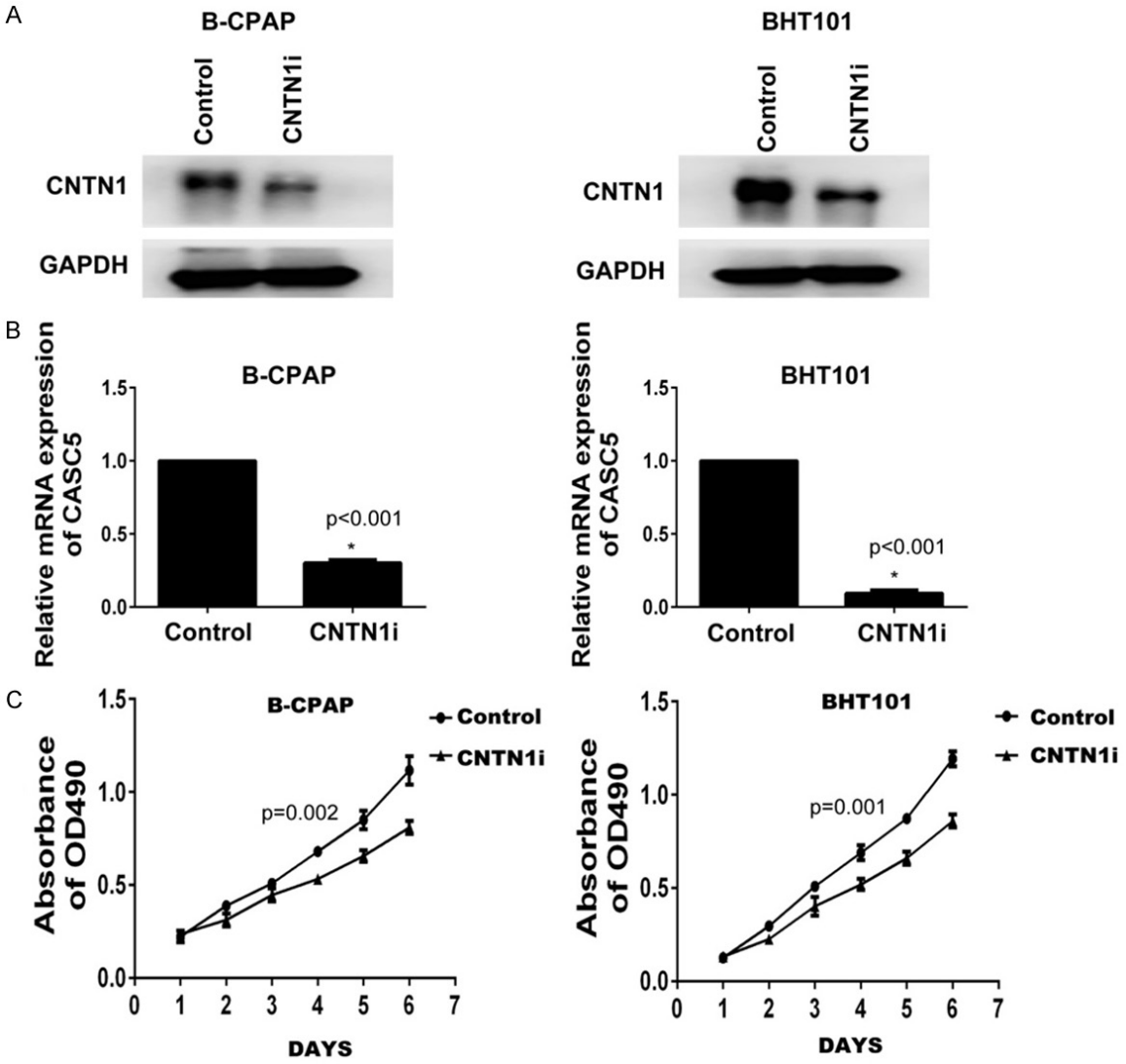

Figure 3. The effect of CNTN1 on the proliferation in thyroid cancer cells. Thyroid cancer cell lines B-CPAP and BHT101 were transfected with 100 nM CNTN1 RNAi or control RNA, then the expression of CNTN1 were detected by Western Blot and Real-time PCR, and the cellular proliferation was assessed by MTT. A. The protein expression of CNTN1 in CNTN1 interfered thyroid cancer cell lines. B. The mRNA expression of CNTN1 in CNTN1 interfered thyroid cancer cell lines. C. The effect of CNTN1 on cellular proliferation in B-CPAP and BHT101 cell lines were detected by MTT proliferation assay. Bars represent the means ± SD of three independent experiments, *P < 0.05.

tional zone which had both normal glandular structure and tumorous structure at one piece of slide (**Figure 2**). By Further analysis, we found there were only 10 paracancer tissues were CNTN1 positive, while 81 cases tumor tissues were positive (**Table 1**).

Correlation of CNTN1 protein expression with clinicopathological characteristics in thyroid cancer

Further analysis was performed in order to explore the role of CNTN1 in the thyroid cancer. From **Table 2**, we could find that CNTN1 level was higher in thyroid cancer with larger tumor size (P = 0.003); and the protein expression of CNTN1 was also significantly correlated with TNM (P = 0.033). 91.9% patient's tumor tissues in III, IV stage possessed positive staining, while 74.6% patients in I, II stage showed positive staining. There were no significant corealtion with age, gender, and LN status.

Knockdown of CNTN1 suppressed the proliferation of thyroid cancer

In order to gain insight into the function of CNTN1 in thyroid cancer, we examined the

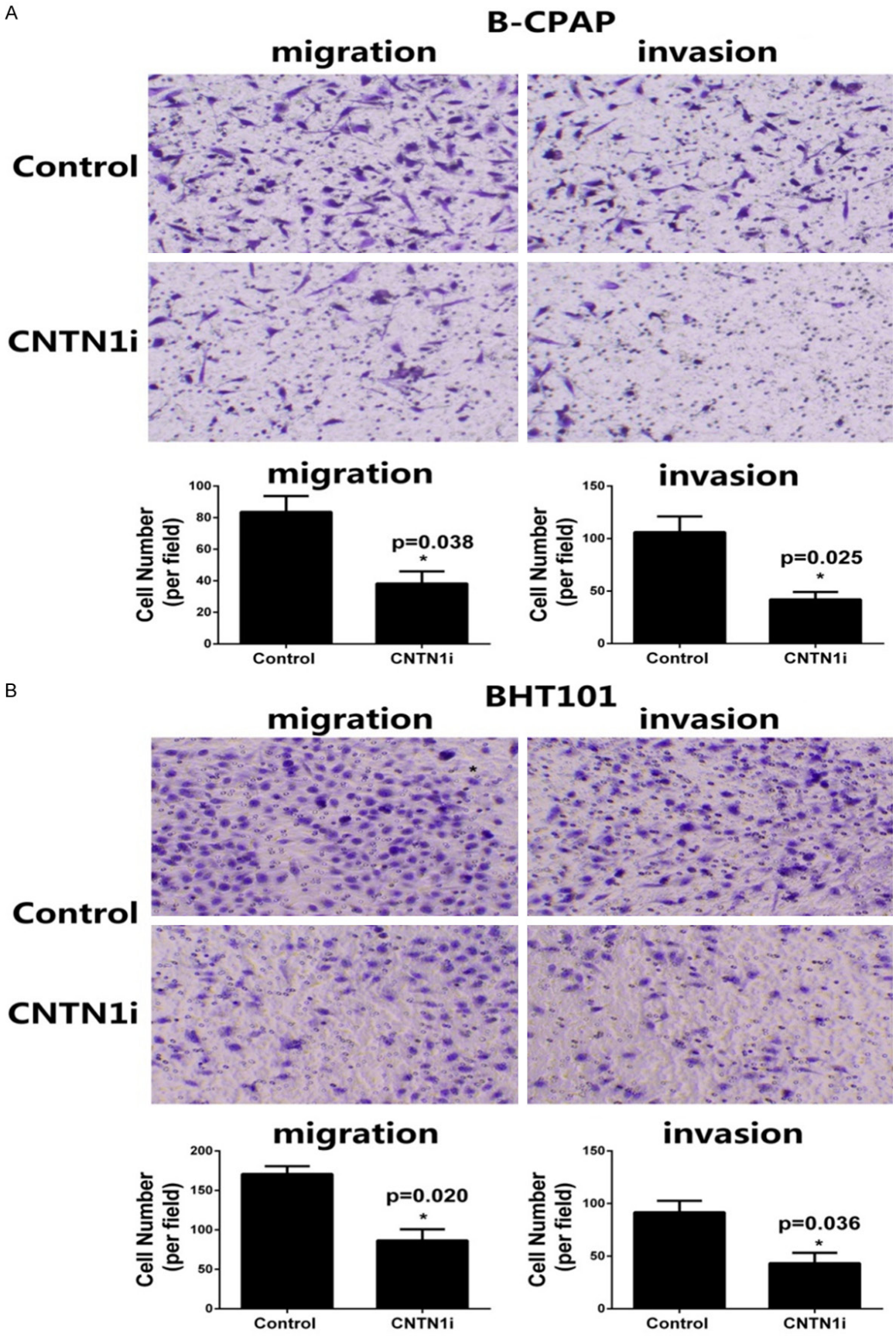
A
B-CPAP
migration
invasion
Control
CNTN1i
migration
Cell Number (per field)
p=0.038
*
Control
CNTN1i
invasion
Cell Number (per field)
p=0.025
*
Control
CNTN1i
B
BHT101
migration
invasion
Control
CNTN1i
migration
Cell Number (per field)
p=0.020
*
Control
CNTN1i
invasion
Cell Number (per field)
p=0.036
*
Control
CNTN1i

Figure 4. The effect of CNTN1 on the migration and invasion in thyroid cancer cells. Thyroid cancer cell lines B-CPAP and BHT101 were transfected with 100 nM CNTN1 RNAi or control RNA, and cellular migration and invasion were evaluated by Transwell assay. A. The migration and invasion in B-CPAP cell line. B. The migration and invasion in BHT101 cell line. All pictures were magnified 100 times, the scale bar size is 200 μm. The results were reproducible in three independent experiments. *P < 0.05.

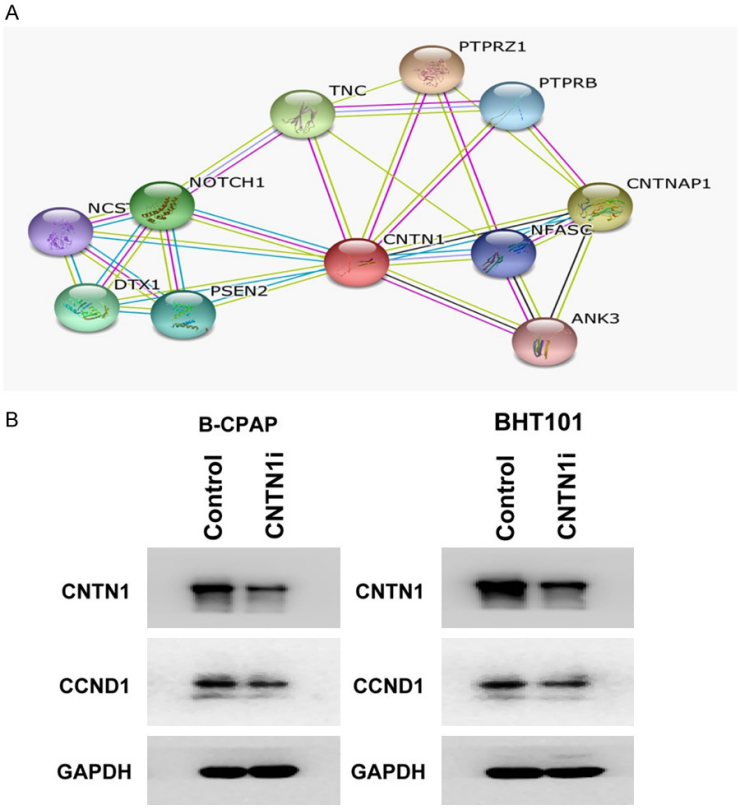

Figure 5. The effect of CNTN1 on Notch pathway. A. Protein-Protein interaction is analyzed by String.10 software. B. Thyroid cancer cell lines B-CPAP and BHT101 were transfected with 100 nM CNTN1 RNAi or control RNA. The expression of CNTN1 and CCND1 were evaluated by Western Blot assay.

change of proliferation after silencing CNTN1 with RNA interference assay (RNAi). Western Blot (**Figure 3A**) and Real time PCR (**Figure 3B**) assays were performed to detect the efficient of RNAi. As shown in **Figure 3C**, thyroid cancer cell lines treated with CNTN1 RNAi complex grew slower than that transfected with control RNA.

Silencing of CNTN1 restrained thyroid cancer migration and invasion

Further, We investigated the effect of CNTN1 on the migration and invasion of thyroid cancer cell lines. As shown in **Figure 4A**, the migration and invasion ability were obviously weakened in B-CPAP cell lines. And this result was also confirmed in another thyroid cancer cell lines BHT101 (**Figure 4B**).

Effects of CNTN1 on notch pathway

In order to further understand how CNTN1 induced cell proliferation and invasion, the potential protein-protein interaction was predicted by String.10 software. As shown in **Figure 5A**, CNTN1 could interact with Notch1. Moreover, we examined the exprssion of one Notch target gene, cyclin D1 (CCND1). As shown in **Figure 5B**, when blocking CNTN1 through interfering assay, we observed the protein expression of CCND1 was significant downregulated.

Discussion

The RET proto-oncogene is a receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor (GDNF) family of extracellular signaling molecules [18]. Mutant and gene rearrangement are prevalent in thyroid carcinoma [19, 20]. And this rearrangement preferentially occurs in papillay thyroid caner among atomic bomb survivors exposed to high radiation dose [17, 21]. Shiozaki et al demonstrates that XB130 plays important role in RET/PTC3 chromosome rearrangement related thyroid cancer cell proliferation and survival [8]. Overactive RET promotes the activation of Ras/Raf/MAPK [7], PI3k and AKT pathway [22]. Inhibitors for RET kinase have been designed and proved effective in the therapy of cancer [23-25]. In this paper, we firstly find the neural adhesion molecular CNTN1 is a new downstream gene of RET/PTC3 fusion gene by the analysis of GEO profile database. This will help us better understand the function of RET rearrangement in thyroid cancer.

Contactins mediate cell surface interactons during nervous system development, and are involved in the signaling between axons and myelinating gial cells via CNTNAP1 [26]. Besides, CNTN1 can act as a ligand of NOTCH1 and promote NOTCH1 activation by releasing notch intracellualr domain (NCID) [12]. Recent studies have reported that overexpression of CNTN1 promotes several cancers metastasis [14, 27]. Liu et al study reveals that VEGF-C can promote the development of esophageal cancer by regulating CNTN1 expression [28]. In this study, we check the level of CNTN1 in 100 pairs thyroid caner. As expected, CNTN1 is significantly evelated in thyroid cancer, moreover, its positive staining is associated with the tumor size and TNM stage. These finding suggest CNTN1 can be a biomarker which is benifit for the diagnosis of thyroid cancer.

The Notch signaling pathway is a highly conserved cell signaling system present in most multicellular organisms [29]. Disoreder of Notch pathway is assocaciated with various cancers which has led to investigation of notch inhibitors as cancer treatments [30]. During the recent years, researchers have proved Notch pathway is also a key regulator of cancer stem cells (CSCs) [31, 32]. As a key ligand of NOTCH1, the function of CNTN1 in thyroid cancer is still unclear. In this study, we firstly prove interferance of CNTN1 obviously depresses cellular proliferation and invasion. We also confirm knockdown of CNTN1 can inhibit the expression of CCND1 .

In summary, our study show that CNTN1 may be a novel effector of RET/PTC3. Abnormity of CNTN1 could be a potential biomarker of thyroid cancer, and participate in the progress of tumor proliferation and metastasis by regulating Notch pathway. This finding may point us a new direction for the diagnosis and treatment of thyroid cancer.

Disclosure of conflict of interest

None.

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