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Original Article

VASN promotes YAP/TAZ and EMT pathway in thyroid carcinogenesis in vitro

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Abstract: Background: Thyroid cancer incidence has been continuity growing globally. To Find reliable molecular biomarkers to assess prognosis and select optimal treatment is necessary. VASN is a protein-coding gene that plays a vital part in tumor development and angiogenesis. Analyzing the TCGA dates, we found VASN could be a potential marker in assessing thyroid prognosis. The act of VASN in thyroid cancer is not explicit. In this article, we investigate the function of VASN expression in thyroid cancer. Methods: The Cancer Genome Atlas (TCGA) unpaired thyroid cancer and normal RNA-seq data was download and our paired thyroid cancer, and a polymerase chain reaction analyzed normal samples. The expression of VASN was regulated by transfected small interfering RNA, and the function of VASN was determined through migration, invasion and cell proliferation assays. Western blot assay was performed to reveal the relation between the VASN expression and YAP/TAZ pathway, epithelial-mesenchymal transition in thyroid carcinogenesis. Results: The significant upregulation of VASN in papillary thyroid carcinoma tissues associated to normal thyroid tissues was revealed by our data and TCGA data. VASN overexpression was significantly correlated to lymph node metastasis, tumor stage and tumor size. In the cell, experiments showed that VASN low expression significantly suppressed the migration, invasion, and proliferation. Western blot assay proves the effect of VASN expression on YAP/TAZ pathway and epithelial-mesenchymal transition. Conclusion: VASN plays a crucial oncogene in thyroid cancer. Our results indicate that VASN could be a biomarker of thyroid cancer and may act in the YAP/TAZ pathway to regulate epithelial-mesenchymal transition (EMT).

Keywords: PTC, VASN, YAP/TAZ, epithelial-mesenchymal transition (EMT)

Introduction

Thyroid carcinoma (TC) has become one of the utmost frequent malignancies of the endocrine system in recent decades [1]. The U.S. National Cancer Institute anticipated 53,990 new cases besides 2,110 deaths numbers of patients due to thyroid carcinoma in the U.S.A in 2018 [2]. An important study also pointed out that global TC cases have increased 4% approximately in each year, and predicted it in 2030 will go beyond colorectal cancer become like the 4th most commonly diagnosed cancer [3]. TC is generally categorized into 4 different histological types, comprising papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), anaplastic thyroid carcinoma, and medullary thyroid carcinoma [4]. Amongst those subtypes, PTC nearly reports for 80%-85% of all cases of thyroid cancer [5]. Ultrasound-guided fine needle aspiration cytology (FNAC) is the foundation of identification of benign disorders and malignancy. Biopsy specimen could use to make gene mutation analysis which was related to thyroid tumorigenesis and prognosis. The detection technology is now surprisingly mature and has been widely used. For example, *BRAF* gene mutation was interested as a marker of aggressive behavior in PTC at the beginning of 2000, which had been already known as a role in other cancers [6]. Some single-center findings, a few metaanalyses, and a large multicenter retrospective worldwide analysis confirmed a correlation between BRAF (V600E) mutation and the probability of recurrence [7-9]. Some scientists studied the mutation in detail, found that BRAF gene mutations activate the MAPK pathway resulting in a loss of control over the cellular cycle, leading to the development of malignancy [10-12]. The detection of BRAF gene mutations is widely used in the biopsy specimen, assisted in diagnosing

papillary thyroid cancer in thyroid nodules, in clinical practice [13, 14]. The growth in the incidence of thyroid malignancy during the last decades without a concomitant growth in mortality might suggest the growing detection of indolent forms of thyroid malignancy and might have driven pointless treatments [15]. It is necessary to lay down an optimized therapeutic strategy, which is related to avoid overtreatment and undertreatment.

VASN, a gene codes a cell-surface protein named vasorin that plays a vital role in tumor development and vasculogenesis [16, 17]. For example, vasorin has been identified as a serum biomarker of hepato-carcinoma, found that highly expressed in sera and tissues [18]. The original of VASN was found as a developmental protein that involves in zebra-fish central neural and vascular morphogenesis and mice embryonic development [19, 20]. It was identified as a part in developing neointima formation after vascular injury and partly regulated the response to pathological stimuli in blood vessel walls [19, 21]. It all known that angiogenesis is essential for tumor progression, and VASN is an indispensable protein mediator between tumor progression and angiogenesis. The high expression of VASN in cancer may promote cancer cell proliferation and migration [22]. It has been reported that VASN could be released in the exosomes from cancer cells and transferred to the recipient endothelial cells promoting migration [16]. We found VASN higher express in PTC than adjacent normal tissues. Nevertheless, the mechanisms underlying VASN in PTC have hitherto not been clarified. Hence, we collected 21 pairs of tumor samples and adjacent normal tissues to validate the expression of VASN by using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Moreover, we also analyzed the data download from TCGA, to found the relationship of VASN expression and clinical features in PTC. We found the VASN expression is associated with tumor size, tumor stage and lymph node metastatic, and could adequately specific diagnose in PTC. In this article, we would like to explore the function of abnormal expression of VASN in thyroid cancer cell lines. All the result of assays supported that VASN plays a crucial role in thyroid oncogenesis, which could be an essential predictive factor for recurrence and metastasis.

Patients and methods

Patients and tumor samples

A total of 21 primary PTCs and matched noncancerous thyroid tissues were achieved at the phase of initial surgery. Samples were snap frozen in liquid nitrogen instantly after lesion resection and stored at -80 degree Celsius before RNA extraction. Entirely specimens were evaluated retrospectively to verify the histological analysis and to confirm abundant malignancy content of the tumor by three pathologists. Major inclusion criteria were: (1) patients with pathologically confirmed PTC in the primary tumor and without any severe diseases in other organs; (2) patients that had received total/near total thyroidectomy and had not received any radiotherapy; (3) patients with a negative history of any other malignant tumors. Major exclusion criteria were: (1) patients with a positive history of other malignant tumors; (2) patients diagnosed with histological types of thyroid cancer other than PTC; (3) patients with severe diseases such as heart failure, stroke, and chronic renal failure; (4) patients with a history of ^{131}I therapy.

Informed consent for the scientific use of biological material was acquired from every patient. The counts of normalized mRNA expression were acquired through the TCGA portal and are expressed as RNA-Seq by transcripts per kilobase million (TPM) values. TCGA clinical information was downloaded in 'Bio-tab' format from the TCGA portal system (https://tcga-data.nci.nih.gov/docs/publications/tcga/). Each patient's data was collected under the protocols permitted by the institutional review board of the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (approval no. 2012-57).

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

By TRIzol reagent according to manufacturer's protocol (Thermo Fisher Scientific) the entire RNA from the cells and tissues were isolated, and ReverTra Ace® qPCR RT Kit (Toyobo, Japan) was used to prepare cDNA. Thunderbird SYBR qPCR Mix (Toyobo, Japan) in the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific) was used to achieve Real-time reverse transcription polymerase chain reaction (qRT-PCR). The primer sequences for PCR

are as below: VASN, forward 5'-CCACCTGCCCTTTGTCCTG-3' and reverse 5'-CAACCTGCCGCTCCTCATT-3'. All loss of function experiments was performed in triplicate. As for an internal control GAPDH was used.

Cell lines and cell culture

TPC1 and BCPAP human thyroid cancer cell lines were obtained from Prof. Mingzhao Xing of the Johns Hopkins University School of Medicine (Baltimore, MA, USA). TPC1 and BCPAP were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen) (Gibco, Grand Island, NY, USA). They were lived in a standard cell culture incubator (Thermo, Waltham, MA, USA) at 37°C with 5% CO_2 and 1 × MEM nonessential amino acids + 1 × sodium pyruvate.

RNA interference

Small interfering RNA (si-RNA) targeting VASN and control si-RNA (si-NC) was supplied by Gene Pharma (Shanghai, China). Using Lipo iMAX (Invitrogen, Grand Island, NY) cells were transfected. Cells were harvested 48 hours after transfection for subsequent RNA expression investigation. All the knockdown experimentations were performed in triplicate. The sequences of si-RNA are as follows:

Si-VASN-1, forward 5'-GCAUGAAAUCACCAAUGAGTT-3', reverse 5'-CUCAUUGGUGAUUUCAUGCTT-3'; Si-VASN-2, forward 5'-CUGGAUGUGAGCAACCUATT-3', reverse 5'-UAGGUUGCUCACAUCCAGCTT-3'; Si-VASN-3, forward 5'-CCGUCUGUGUCAUGCCUUUTT-3', reverse 5'-AAAGGCAUGACACAGACGGTT-3'.

Cell colony formation assay

Colony formation assay was achieved via monolayer culture. The designated cells were seeded into six-well plates at 1500 cells/well (TPC1), 1500 cells/well (BCPAP). After 5-7 days of culture, surviving colonies were fixated with 4% PFA (paraformaldehyde, Sigma, USA) for 15 mins and stained with 0.01% crystal violet for 15 mins, and later, an image was captured by using a camera. All colony formation assays were performed in triplicate.

Transwell migration and invasion assay

Cell migration and invasion were assessed using 24-well Transwell plates (8 μm pore size, Corning Co., Ltd., USA). Three hundred microliters of serum-free medium containing 4×10^4 cells from each subgroup were added to the upper chamber (Corning Costar Corp., Cambridge, MA, USA), and 600 μl of medium containing 10% FBS was added into each lower chamber. After incubation at 37°C for 24 h, the cells remaining on the upper membranes were removed with a cotton swab. The cells that had migrated or invaded through the filters were fixed with 4% paraformaldehyde and stained with 0.01% crystal violet. Cells in five random fields were counted under a microscope at 200 × magnification. We used BioCoatTM Matrigel Invasion chamber 24-well Plate 8.0 Micron (Corning, NY, USA). The same treatment was conducted to invasion assay. The experiments were independently performed in triplicate.

Cell apoptosis assay

An Annexin V/propidium iodide (PI) apoptosis kit (Nanjing Key Gen Biotech. Co., Ltd., Nanjing, China) were applied to regulate the apoptotic rates of TPC1, BCPAP as suggested by to the company's advice. The cells were collected, rinsed three times by PBS, and suspended in 1 × binding buffer (Beyotime Institute of Biotechnology, Haimen, China) at a concentration of 1×10^6 cells/ml. Cell suspensions of 300 μl were stained with 3 μl of Annexin V-fluorescein isothiocyanate and 3 μl of PI at room temperature for 15 minutes in the darkroom prior to analysis by flow cytometry (BD FACS Aria; BD Biosciences, Franklin Lakes, NJ).

Western blot analysis and antibodies

Total protein lysates were parted by 10% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (BioRad, Berkeley, CA, USA) and electrophoretically transported to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blotted with 5% non-fat milk for in TBST 2 hours at room temperature, then, washed 3 times by TBST and probed with mouse anti N-cadherin (cat no. 66219-1-Ig; Proteintech, Inc; 1:2,000 dilution), vimentin (cat no. 60330-1-Ig; Proteintech, Inc; 1:2,000 dilution), E-cadherin (cat no. 60335-1-Ig; Proteintech, Inc; 1:2,000 dilution), TAZ (cat no. 66500-1-Ig; Proteintech, Inc; 1:1000 dilution), YAP1 (cat no. 66900-1-Ig; Proteintech, Inc; 1:1000 dilution), Rabbit-VASN (cat no. YN2042; ImmunoWay Biotechnology, Inc; 1:1000 dilution) and mouse-β-actin (cat no. 66009-1-Ig; Proteintech, Inc; 1:2,000 dilution) according to the manufacturer's protocol over-

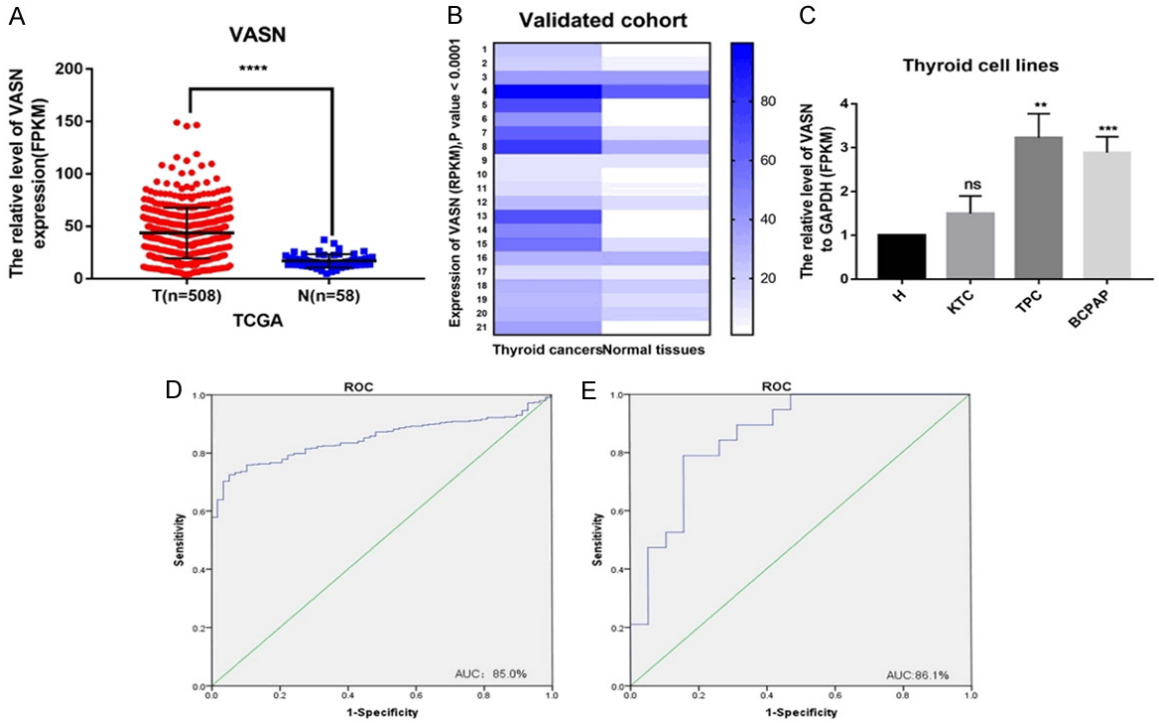

Figure 1. VASN expression in Thyroid cancer in the TCGA cohort validated cohort and thyroid cancer cell lines. A. The TCGA cohort contained 508 thyroid cancer tissues and 58 normal tissues. The analysis was done using the unpaired t-test. ****P<0.0001. B. A hot map was used to describe the VASN expression examined by RT-qPCR in 21 paired thyroid cancer tissues and adjacent noncancerous tissues (paired t-test, P<0.0001). C. The relative expression of VASN gene using RT-qPCR in thyroid cancer lines, VASN was expressed at a higher level in TPC, BCPAP. D. ROC curve for VASN expression to diagnose thyroid cancer in the TCGA cohort. The AUC was 85.0% (81.7-88.3%). E. ROC curve for VASN expression to diagnose thyroid cancer in the validated cohort. The area under the ROC curve (AUC) was 86.1% (74.4%-97.9%).

night at 4°C. The membranes were then incubated with the anti-mouse IgG or anti-rabbit IgG as a secondary antibody for 1.5 hours at room temperature.

Statistical analysis

Statistical data evaluations were accomplished using SPSS 23.0 software (SPSS, Inc., Chicago, IL). GraphPad Prism7 (GraphPad Software, NC., La Jolla, CA, USA) was used for creating the graph images. Student's t-test was applied for analyzing two group comparisons; With one-way ANOVA multiple group comparisons were analyzed. P<0.05 was considered to specify a statistically significant difference.

Results

VASN expression is up-regulated in PTC tissues and cell lines

To investigate the biological purpose of VASN in thyroid cancer. In our study, we assessed the VASN expression in the TCGA and measured the levels of VASN in thyroid cancer tissues and corresponding normal tissues by RT-qPCR (21 pairs). We found VASN expression is significantly upregulated in thyroid cancer. TCGA (Thyroid cancers tissues 43.55 ± 1.077, n=502: Normal tissues 17.07 ± 0.8229, n=58, unpaired t-test P<0.0001) (**Figure 1A**). A heat map was made to observe the differential expression in collected surgical tissue samples (21 pairs thyroid cancer tissues and paired normal tissues, Δd=-25.39 ± 21.5, paired t-test, P<0.0001) (**Figure 1B**). It suggests that VASN could be a potential marker to monitor thyroid cancer in a variety of thyroid cancer cell lines, the expression of VASN also performs a higher level compared with normal cells line (compared to HTORI-3, TPC, P<0.01; BCPAP, P<0.001) (**Figure 1C**).

The overexpression of VASN gene could predict PTC

We have shown that VASN expression is elevated in cancer. Then, a receiver operator characteristic (ROC) curve was plotted to establish the

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

Clinicopathologic characteristics		High expression	Low expression	*P* value
Age	≤60	241	108	0.051*
	>60	59	40	
Gender	Female	83	40	0.49
	Male	217	108	
Stage	I/II	184	106	0.020*
	III/IV	116	42	
Tumor size	T1-2	169	100	0.014*
	T3-4	131	48	
Lymph Node	N0	124	103	0.001*
	Nx	176	45	
Status	Alive	294	141	0.096
	Dead	6	7	

*P<0.05.

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

Clinicopathologic characteristics		High expression (%)		*P*-value
Age	≤45	3	5	0.052
	>45	10	2	
Gender	Female	6	4	0.5
	Male	7	3	
Stage	I/II	7	6	0.177
	III/IV	6	1	
Tumor	T1-2	5	3	0.608
	T3-4	8	4	
Lymph Node	N0	6	4	0.63
	Nx	7	3	

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

Clinicopathologic features	OR	95% CI	*P*-value
Age	0.664	0.422-1.043	0.075
Gender	0.657	0.433-0.998	0.049*
Stage	3.535	2.342-5.336	<0.001*
Tumor	2.655	1.797-3.923	<0.001*
VASN expression	3.249	2.137-4.939	<0.001*
Status	1.668	0.537-5.178	0.376

*P<0.05.

diagnostic value of VASN expression. We achieved AUC 85.0% (81.7-88.3%) for the TCGA cohort (**Figure 1D**) while AUC 86.1% (74.4%-97.9%) in the validated cohort (**Figure 1E**). This indicated that the expression of VASN had high reliability in the identification of PTC.

VASN expression is related with tumor size, tumor stage and lymph node metastatic

Analyzed clinical feature of the 508 thyroid cancer tissues from TCGA, patients were group by median value into Low expression and High expression. We found the tumor stage (P=0.02<0.05), tumor size (P<0.014) and the lymph node metastasis (P<0.001) were significantly differenced in higher expression group vs. lower group (**Table 1**). Due to a smaller number of samples to yield reliable results, there isn't have statistical significance in our validated cohort (**Table 2**).

Overexpression of VASN represents an increased risk of lymph node metastasis

To examine whether the expression level of VASN is related with lymph node metastasis, by logistic regression we examined the relationship between them (**Table 3**). Univariate logistic regression analysis suggested that the significant variables for lymph node metastasis were VASN expression (odds ratio [OR] 3.249, 95% CI 2.137-4.93, P<0.001), clinical stage (OR 3.535, 95% CI 2.342-5.33, P<0.001), gender (OR 0.657 95% CI 0.433-0.998, P=0.049), tumor size (OR 2.655, 95% CI 1.797-3.923, P<0.001 (**Table 4**). Multivariate logistic analysis revealed that VASN expression (OR=3.106, 95% CI 2.001-4.822, P<0.001), clinical stage (OR=2.702, 95% CI 1.674-4.359, P<0.001), tumor size (OR=1.554, 95% CI 0.978-2.470, P=0.062) and gender (OR=0.741, 95% CI 0.470-1.168, P=0.197) were associated with lymph node metastasis. That's to say, VASN expression serves as a risk of lymph node metastasis.

Down-regulation of VASN inhibited migration and invasion

Invasion and migration assay often use to evaluate tumor metastasis capacity. We found that downregulating the expression level of VASN

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

Clinicopathologic features	OR	95% CI	*P*-value
Gender	0.741	0.470-1.168	0.197
Stage	2.702	1.674-4.359	<0.001*
Lymph Node	1.554	0.978-2.470	0.062
VASN Expression	3.106	2.001-4.822	<0.001*

*P<0.05.

inhibited the TPC1, BCPAP migrate (**Figure 4A**) and invade (**Figure 4B**). From the upper chamber to the bottom chamber, compared with the control group. In a word, there is a positive correlation between the VASN expression and thyroid cancer metastasis capacity.

Downregulation of VASN inhibited proliferation in thyroid cancer cell lines

We use the small interference RNA (siRNA) to silenced VASN (**Figure 2A**). To compare with the control group, we choose two effective silence group (Si-VASN-1, Si-VASN-2) to complete the following experiments. We assessed the ability of cells growth by CCK-8 proliferation assay (**Figure 3A**, **3B**). And Colony forming assay (**Figure 3C**), we found that downregulating the level of VASN expression significantly inhibit proliferation in thyroid cancer cell line (TPC1, BCPAP).

VASN promotes an EMT in thyroid carcinogenesis via triggering oncogenic YAP/TAZ pathway

The Hippo pathway has been described to restrict tissue growth and modulate cell differentiation, proliferation, and migration. YAP and TAZ were two major downstream effectors of the Hippo pathway. There was experimentally documented that YAP activation leads to EMT pathway activated that promoted cancer cell invasion and metastasis. After knockdown VASN in PTC cell lines, we found the YAP/TAZ pathway inhibition that results in a reduction of the mesenchymal marker N-cadherin, vimentin, and excitation of the epithelial marker E-cadherin (**Figure 5**).

Discussion

Since the 1970s, the occurrence of thyroid carcinoma increased by almost 3.5 fold [23, 24]. Thyroid carcinoma is expected to surpass colorectal cancer and became the fourth leading cancer diagnosis in 2030 [3]. However, with the development of economy and technology, people's request for improving the life quality and healthy standard becomes higher. Surgery is the primary management for papillary thyroid cancer [25]. In recent years, some experts put forward that conservative treatment should be considered in low-risk PTC. How to screen for high-risk patients and filter for low-risk is a crucial point of future therapies. The tumorigenesis is mainly decided by genomic variation. Different molecular biomarkers could predict the clinical progress and metastasis of PTC. Finding reliable molecular biomarkers assess the degree of PTC risk is indispensable.

VASN has been proved a key mediator of communication between tumor cells and endothelial cells [16]. Accumulating evidence has confirmed that VASN has a crucial function in the process of Human Umbilical Vein Endothelial Cells Migration promote hepatocellular carcinoma growth. But regulate the expression of VASN in hepatoma carcinoma cell in vitro also could inhibit invasion. However, the underlying molecular mechanisms of VASN in vitro are still weakly understood.

In our present research, we found the potential of VASN to be an oncogene in PTC. So, we analyzed the TCGA date the outcomes revealed that VASN expression was overexpressed in tumor tissues, which support the assumption. Then, we chose 21 matched pairs of PTCs to extract RNA and qRT-PCR. The outcomes revealed that VASN expression was significantly different between cancer tissues and adjacent normal tissues. This finding suggested VASN potential role as an oncogene in PTC. In the TCGA cohort and validated cohort, the clinicopathologic feature analysis presented that the overexpression of VASN was significantly associated to the aggressive clinicopathologic characteristics, such as tumor size, tumor stages, and LNM. The loss-of-function study indicated that VASN knockdown could inhibit cell proliferation and significantly impaired the migration and invasion in vitro.

The Hippo pathway was originally recognized in Drosophila melanogaster screens for tissue growth 20 years ago. In humans, the primary purposes of the Hippo pathway have been described to restrict tissue growth and modulate cell differentiation, proliferation, and mi-

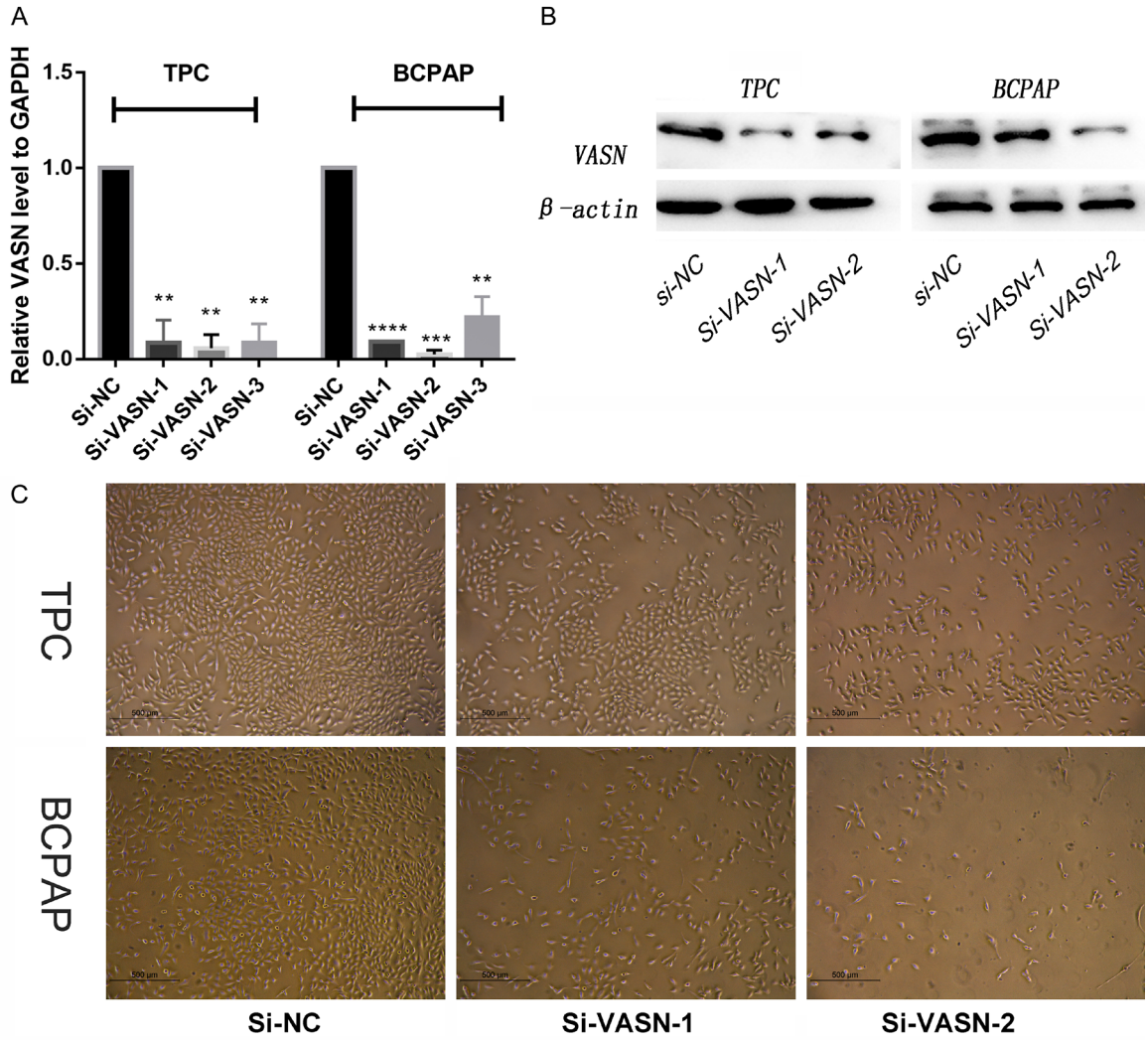

Figure 2. Down-regulated VASN gene expression in thyroid carcinoma cells. A. TPC and BCPAP cells were transfected with siRNA-NC, si-VASN-1, si-VASN-2 and si-VASN-3 for 48 h and then analyzed by PCR to revealed mRNA levels of VASN expression in these lines. Compared with the corresponding control group, the knock-down of VASN gene in si-VASN-1 and si-VASN-2 group was effective. B. TPC, BCPAP cells were transfected with si-VASN-1 or si-VASN-2 or si-NC for 48 h, and protein levels of VASN are significantly reduced. C. Observed the cytomorphosis after knockdown VASN under a microscope.

gration [26]. The Yes-associated protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ), two major downstream effectors of the Hippo pathway [27]. YAP is a potential oncogene, that expression encouraged epithelial-mesenchymal transition eliminated apoptosis and promoted proliferation [28]. YAP expression is too associated to oncogenic properties in numerous human cancers for example hepato-cellular cancer, non-small cell lung cancer, breast cancer, esophageal squamous cell cancer, ovarian cancer and gastric cancer [28-32]. Our results demonstrated that silencing of VASN would inhibit the expression of YAP/TAZ in protein levels.

It has been experimentally well-known that over-expression of YAP lead to in a decrease of the epithelial marker E-cadherin and phenotypic alteration that is correlated with epithelial-mesenchymal transition (EMT), promoting cancer cell invasion and metastasis [28, 33]. EMT, a trademark of tumorigenic transformation, is a cellular program by which epithelial cells lose cell-cell adhesion and obtain mesenchymal traits [34]. The common biomarkers in EMT pathway comprising suppression of epithelial markers (for example; E-cadherin) and up-regulation of mesenchymal markers (for example; N-cadherin, Vimentin) can promote tumor metastasis [35]. There is a report put forward that

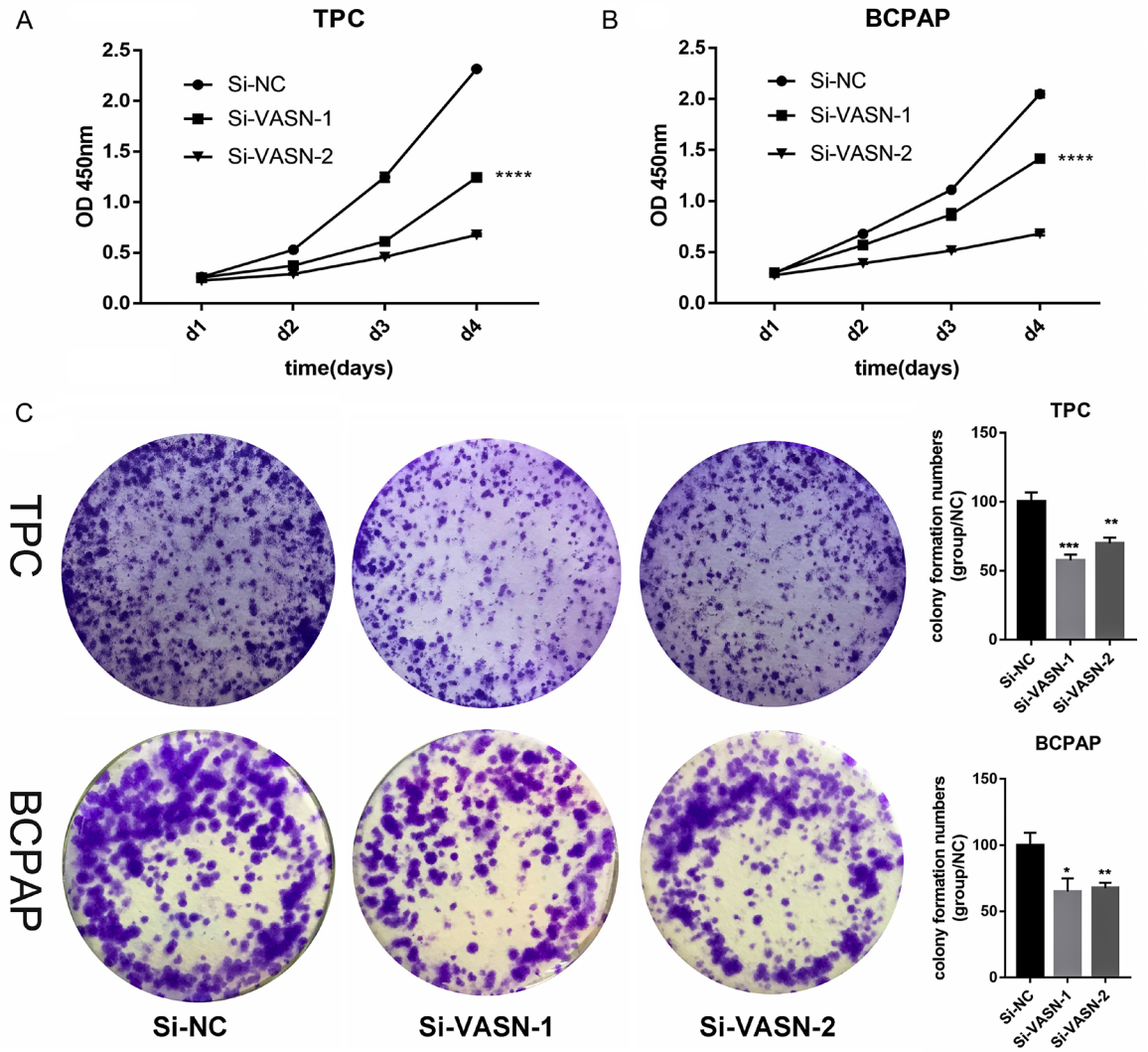

Figure 3. Down-regulated VASN expression in thyroid carcinoma cells inhibited proliferation and colony formation. Cell proliferation assay: knockdown of VASN in TPC (A) and BCPAP (B) cells compared with the corresponding control group, cell proliferation was measured using CCK-8. Colony formation assay: TPC and BCPAP cells (C) transfected were cultured in 6-well plates for 10-14 days. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

CagA+ *H. pylori* infection further promotes activation of the YAP pathway signaling that enhances EMT via activation, thus promoting cell migration and gastric tumorigenesis [36]. We demonstrated silencing of VASN inhibit not only the expression of YAP/TAZ but also N-cadherin, vimentin, and excitation of E-cadherin as we suspected before. Due to limited experimental conditions, we did not further explore the direct interaction between the two pathways.

Conclusion

In summary, our study results showed that we found VASN as a new potential marker for the treatment and diagnosis of PTC. And proposing a possible theory that VASN promotes an epithelial-mesenchymal transition in PTC via triggering oncogenic YAP/TAZ pathway.

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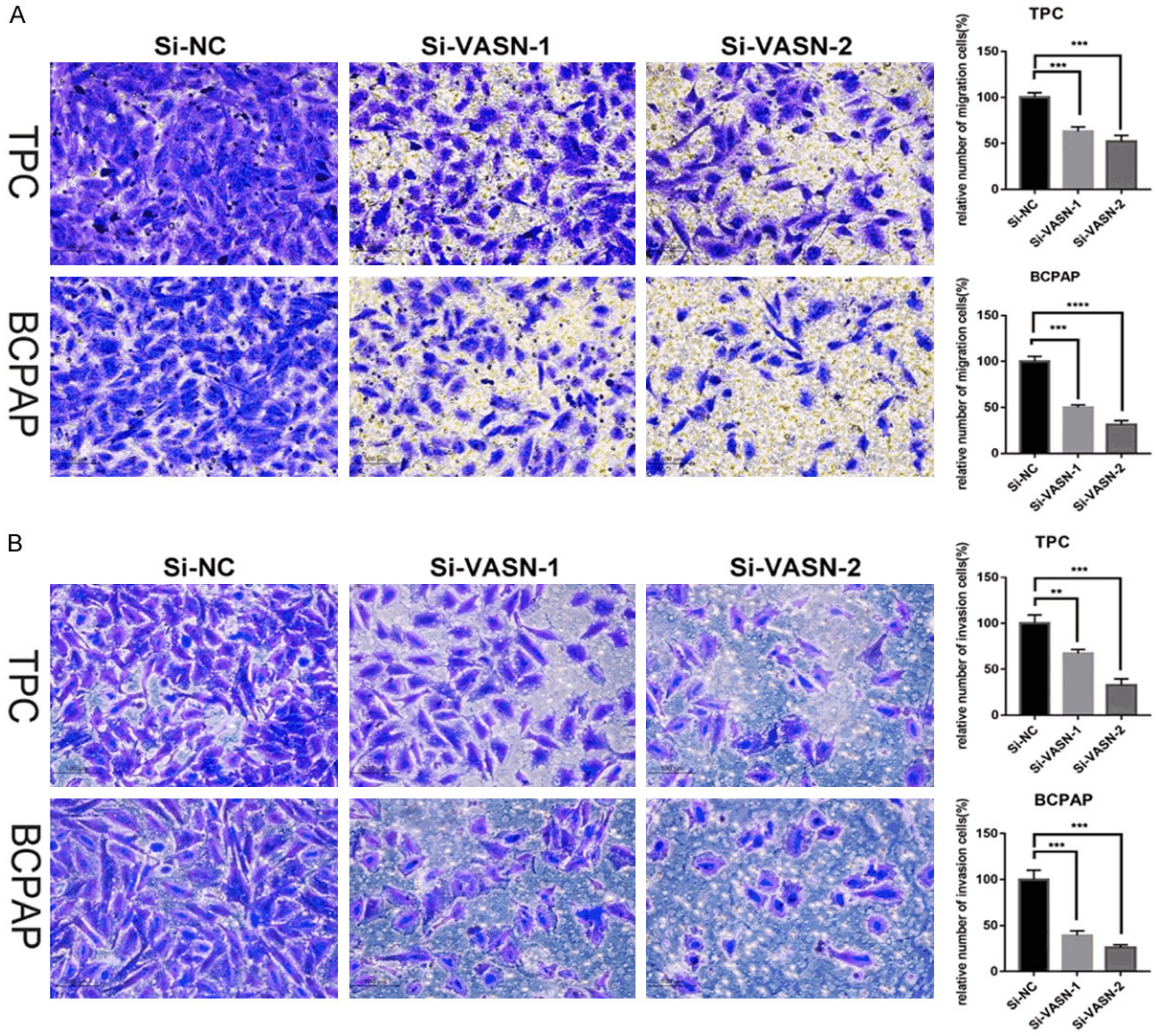

Figure 4. Downregulation of VASN gene expression in TPC and BCPAP cells inhibits migration and invasion. A. Migration assay. Migrating cell number was observably less in TPC and BCPAP cells after down-regulated VASN expression compared with NC. And relative quantification of the migrating cell number. B. Invasion assay. Invading cell number was observably less in TPC and BCPAP cells after down-regulated VASN expression compared with NC. And relative quantification of the invading cell number. The stained cells were then counted manually from 3 randomly selected fields and normalized with cell proliferation. Comparison with the NC group using Student's t-test. **P<0.01; ***P<0.001, ****P<0.0001.

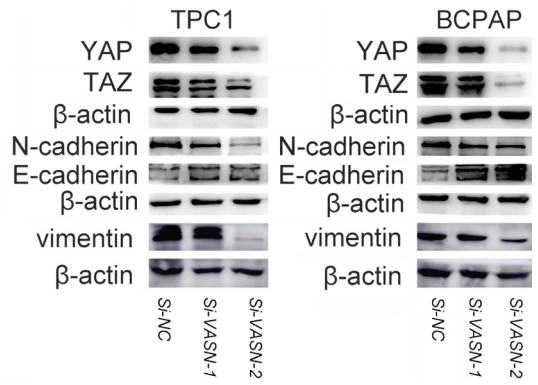

Figure 5. Downregulation of VASN gene expression in TPC and BCPAP cells inhibits the Hippo pathway and EMT pathway. YAP, TAZ, N-cadherin, E-cadherin, and Vimentin protein expression determined by western blotting. All samples were total unified total proteins by β-actin. The pictures were taken under a microscope before protein extraction.

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Consent for publication was obtained from all participants. The patients issued written in-

formed consent for the publication of this research and accompanying images. A copy of the written consent is ready for review by the Editor in Chief of this journal.

Disclosure of conflict of interest

None.

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