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ORIGINAL ARTICLE

ZNF703 is Overexpressed in Papillary Thyroid Carcinoma Tissues and Mediates K1 Cell Proliferation

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Abstract

Zinc finger protein 703 (ZNF703), a member of the NET family of transcription factors, has recently emerged as an important player in the development of several types of cancers, though its role in papillary thyroid cancer (PTC) has not been characterized. We investigated the expression of ZNF703, its association with the most common genetic mutation in PTC, BRAF V600E, and its potential use as a therapeutic target. Real-time PCR, immunohistochemical staining, and western blot analysis of ZNF703 expression were performed for 36 cases of PTC and corresponding normal thyroid tissues. ZNF703 mRNA and protein expression was found to be significantly higher in PTC compared to normal thyroid tissues ($P < 0.05$). Furthermore, expression was associated with the tumor size, lymph node metastasis, and advanced disease stage. Immunohistochemical results showed that there was no correlation between ZNF703 protein levels and BRAF V600E mutation. The human PTC cell line K1, which has a BRAF V600E mutation, was selected for further investigation. Using small interfering RNA (siRNA), ZNF703 was shown to contribute to the proliferation, apoptosis, and invasion of K1 cells. ZNF703-siRNA downregulated E2F1 and MMP9 protein expression and enhanced the expression of p27 protein ($P < 0.05$), but had no effects on BRAF V600E protein levels. These results suggest that ZNF703 may be of potential use as a new marker for PTC prognosis and therapy that functions independent of BRAF V600E expression.

Keywords ZNF703 · Papillary thyroid cancer · siRNA · Proliferation · Apoptosis · Invasion

Introduction

Thyroid carcinoma represents the most common endocrine malignancy in humans [1]. Its incidence is increasing worldwide, and by 2030, it is expected to be the fourth most common cancer [2]. Papillary thyroid carcinoma (PTC) primarily accounts for the increasing incidence of thyroid cancer [3, 4]. Epidemiological studies suggest that exposure to pollutants [5–7], ionizing radiation [8–10] and excessive dietary iodine [11, 12] and insulin-resistance [13–15] contribute to PTC. Epidemiological studies also indicate that PTC is more prevalent in women than in men, which suggests that estrogen may be involved in its development [16–19].

In PTC and other types of cancer, genetic and molecular abnormalities have been associated with tumorigenesis. Aberrant expression or function of multiple oncogenes contributes to oncogenesis and metastasis in human cancers. The $BRAF^{T1799A}$ mutation, a thymine to adenine transversion at nucleotide 1799, which involves a valine to glutamate substitution at residue 600 (V600E), is the most common genetic event in PTC. The mutation continuously activates the RAS/RAF/MEK/ERK/MAPK pathway. It has been reported that approximately 45–60% of PTCs have BRAF V600E mutations [20]. Various molecular detection methods are available to determine BRAF mutation, including next-generation sequencing (NGS) and mutation-specific PCR [21]. Because these DNA-based techniques are complex and the detection period is long, immunohistochemistry (IHC) with anti-BRAF V600E antibody has become a new sensitive and specific method for detecting BRAF V600E mutation [22, 23]. Although BRAF V600E inhibitors have brought promising results in clinical trials to PTC, most patients eventually develop resistance to BRAF V600E inhibitors [24, 25]. However, tumor genesis involves changes in complex molecular networks, highlighting the importance of seeking other molecular targets.

ZNF703, a member of the NET family of transcription factors, has been implicated in the regulation of a wide variety

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of developmental processes [26, 27]. Abnormally expressed ZNF703 has been reported in various tumors, with its expression correlating with tumor progression [28–33]. Several studies have identified ZNF703 as a target gene of estrogen receptor (ER) transcriptional activity [29]. However, the role of ZNF703 in PTC, as a tumor related to estrogen, and the possible association of ZNF703 with BRAF mutation, have not been reported.

In this study, we assessed the expression of ZNF703 in PTC and evaluated the correlation between its expression and clinicopathological features of PTC patients. We further detected the expression of the mutated BRAF V600E protein and its correlation with the expression of ZNF703 in the same cases. To characterize the role of ZNF703 in PTC, we analyzed the biological consequence of siRNA-mediated knockdown of ZNF703 expression in cultured PTC cells and identified downstream regulatory genes that are modulated in response to ZNF703 knockdown. These studies provide a molecular basis for the function of ZNF703 in PTC, as well as a potential new target for therapeutic intervention.

Table 1 Association of ZNF703 protein expression with clinicopathological features from papillary thyroid cancer patients

Characteristics	NO.	ZNF703			*p*
		+	–	%	
Gender					
Male	6	6	0	100	0.079
Female	30	18	12	60.0	
Age (y)					
≥45	15	9	6	55.3	0.175
<45	21	15	6	76.2	
Tumor size (cm)					
≤4	21	11	10	52.4	0.040
>4	15	13	2	86.7	
Lymph node metastasis					
+	8	8	0	100	0.033
-	28	16	12	57.1	
Disease stage					
I, II	27	15	12	55.6	0.016
III, IV	9	9	0	100	

Materials and Methods

Tissue Specimens and Cell Culture

A total of 36 patients with histologically confirmed papillary thyroid cancer in Tangshan Gongren Hospital and Tangshan Renmin Hospital from 2010 to 2015 were selected for this study. Fresh tumor tissues and corresponding normal thyroid tissues were obtained during routine surgery and were stored in liquid nitrogen for RT-PCR and western blot assays, or they were fixed in 4% paraformaldehyde solution and then embedded in paraffin for immunohistochemical staining. The clinicopathological features of the patients are detailed in Table 1. The study was approved by the Ethical Committee of Tangshan Gongren Hospital and all patients provided written informed consent.

Human PTC-derived K1 cells were obtained from The European Collection of Authenticated Cell Cultures and cultured in RPMI 1640 containing 10% fetal calf serum (Gibco Co., Grand Island, NY, USA) at 37 °C in a 5% CO_2 humidified atmosphere. BRAF V600E mutation is the driver of oncogenic mutation of K1 cells [34].

Immunohistochemical Staining

Four-micrometer sections of paraffin-embedded tissues were conventionally dewaxed and then repaired in a pressure cooker at 100 °C for 2 min. Next, 3% H_2O_2 was added for 10 min to eliminate endogenous peroxidase, followed by 20% normal goat serum at 37 °C for 20 min and rabbit anti-ZNF703 antibody (1:50, ABCAM Technology, St. Louis Park, MN, USA) or mouse anti-BRAF V600E protein (1:50, Spring Bioscience, Pleasanton, California) at 4 °C overnight. The next day, the sections were washed twice with phosphate-buffered saline (PBS) and then incubated with a horseradish peroxidase-conjugated secondary antibody (Zhongshan Jinqiao Biotech, Beijing, China) at 37 °C for 30 min. The sections were then washed with PBS three additional times and incubated with diaminobenzidine solution (Zhongshan Jinqiao Biotech) as the chromogen to visualize the positive signal. The sections were counterstained with Mayer's hematoxylin for 60 s and dehydrated in upgraded ethanol solutions, clarified with xylene and sealed with cover slips. The negative control section was processed as above but using PBS instead of the primary antibody. ZNF703 expression was determined based on the percentage of positive cells, combined with the staining intensity. The percentage of positive cells was divided into four levels: 0 point: ≤ 10% of positive cells, 1 point: 11–30%, 2 points: 31–50%, and 3 points: > 50% of positive cells. The intensity of staining was classified as: 0 point: no staining; 1 point: weak staining (light yellow); 2 points: moderate staining (yellowish-brown); and 3 points: strong staining (brown). The final score of ZNF703 expression was calculated as the sum of the ZNF703 expression rate and intensity scores, with ≥2 considered positive. Cells within five randomly selected fields of each core were counted under a microscope (10 × 20). According to previously published methods, tumor tissue was considered positive for mutation BRAF V600E if it displayed a staining intensity of 3 points irrespective of the number of tumor cells stained [23].

SiRNA and Transfection

Human ZNF703 siRNA (sense strand, 5'-AGGACAAG UCCAGCUUCAAGCCCUATT-3; antisense strand, 5'-UAGGGCUUGAAGCUGGACUUGUCCUTT-3′) and negative control siRNA (nonsilencing siRNA) were purchased from HanHeng RNAi Company (Shanghai, China). Non-transfected cells were used as blank control group. K1 cells were seeded in 6-well culture plates at a density of 3×10^5 cells. After incubation overnight, the cells were transiently transfected with ZNF703-siRNA (2.5 μg/well) or the nonsilencing siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The ability of ZNF703-siRNA to inhibit ZNF703 mRNA and protein expression was analyzed by RT-PCR and western blotting.

Real-Time PCR Analysis

RNA was isolated from the 36 paired PTC and non-cancerous thyroid tissue samples, or from transfected K1 cells using Trizol reagent (Invitrogen) as per the manufacturers' instructions. After verification of purity and concentration, the RNA was transcribed into cDNA using the cDNA Synthesis kit (Invitrogen). The cDNA was subjected to RT-PCR, using the SYBR Green PCR Supermix kit (Invitrogen) with the Rotor gene-3000 instrument (Corbett). Reactions were performed in 20 μL volumes with 1 μL cDNA. Primer sequences for ZNF703 were 5'-AACGGCCCACATGAGTCAAT-3′ and 5'-GGCGGGGATCATGTCGTTAT-3′. Primer sequences used for GAPDH were 5'-GAAAGCCTGCCGGT GACTAA-3′ and 5'-AGGAAAAGCATCACCCGGAG-3′. The PCR protocol was as follows: 95 °C for 2 mins and then 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH serving as a reference gene for normalization.

Western Blot Analysis

Protein was isolated from the 36 paired PTC and non-cancerous thyroid tissue samples and/ or from K1 cells 72 h after transfection by dissolving in RIPA buffer (Beyotime Biotech Institute, Shanghai, China) with phenylmethylsulfonyl fluoride. The concentration of total cellular protein was measured using a BCA kit (Beyotime Biotech). Aliquots of 25 μg of protein extracts were electrophoresed on 10% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were incubated overnight at 4 °C with primary antibodies against ZNF703 (1:300, ABCAM), BRAF V600E (1:300, NewEast Biosciences, King of Prussia, PA, USA), E2F1 (1:500, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), p27 (1:500, Santa Cruz), MMP9 (1:500, Santa Cruz), or β-actin (1:500, Santa Cruz). The membranes were washed and probed with anti-mouse or anti-rabbit horseradish peroxidase-linked secondary antibody (Beyotime Biotech) and visualized with diaminobenzene detection reagent (Beyotime Biotech). The protein bands were scanned and quantified using Quantity One Software (Bio-Rad). Expression of β-actin protein served as a loading control.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl Tetrazolium Bromide (MTT) Analysis

The MTT assay was used to assess K1 cell proliferation after transfection with ZNF703-siRNA. K1 cells (10^4 per well) were seeded in 96-well plates. At 0, 24, 48, 72, and 96 h post-transfection, cell viability was determined. Ten microliters of MTT solution (5 mg/mL; from Sigma, St Louis, MO, USA) was added, and the cells were incubated for 4 h. Next, the cell culture medium was removed and 150 μL of dimethyl sulfoxide (Sigma) was added to dissolve the MTT. Absorbance at 490 nm was measured using a microplate reader (Bio-Rad, Richmond, CA). The cell culture medium without siRNA treatment served as the negative control.

Flow Cytometric Analysis

The effect of ZNF703-siRNA on the apoptosis of K1 cells was determined by Flow Cytometric Analysis. Cells of each group at 72 h post transfection were trypsinized and collected by centrifugation at 800 r/min for 5 min. They were then incubated at room temperature for 15 min with 0.5 mL of binding buffer and 1 μL AnnexinV-FITC from the Annexin V-FITC apoptosis detection kit (Merck, Darmstadt, Germany). After that, the cells were resuspended in 0.5 mL fresh binding buffer containing 5 μL propidium iodide (PI), and then, apoptosis was measured by the FACScalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Hoechst 33342 Analysis

For cell apoptosis analyses, K1 cells (72 h after transfection) were collected and washed with PBS. Then Hoechst 33342 (10 μg/mL, Sigma) was added directly to the culture medium. After 15 min incubation at room temperature in the dark, the nuclear morphology of the cells was observed with a confocal laser scanning microscope.

Transwell Invasion Assays

For invasion assays, a total of 3×10^5 K1 cells in serum-free medium were seeded on a matrigel-coated polycarbonate membrane inserted in a transwell apparatus (BD Biosciences). After incubation for 13 h, the cells adhering to the lower surface were fixed with methanol, stained with

Giemsa solution, and counted under a microscope on five randomly selected fields.

Statistical Analysis

All statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). The χ^2 test was used to analyze differences in ZNF703 immunohistochemical staining between the PTC tumors and normal thyroid tissues. The correlation between ZNF703 and mutation BRAF V600E expression was assessed by the Spearman test. Other statistical data are presented as means ± standard deviation. Single-factor analysis of variance (one-way ANOVA) was used to compare multiple samples. A P value less than 0.05 was considered statistically significant.

Results

Overexpression of ZNF703 in Thyroid Tissues

To determine whether ZNF703 is overexpressed in PTC, we performed RT-PCR of ZNF703 mRNA in 36 PTC tissues and corresponding normal thyroid tissues. ZNF703 mRNA expression was significantly increased in PTC tissues ($P < 0.05$, Fig. 1a). We further assessed ZNF703 protein expression in the same tissues. ZNF703 protein expression was significantly increased in PTC tissues as assessed by western blotting ($P < 0.05$, Fig. 1b). Immunohistochemistry results showed that ZNF703 was localized in the nucleus of PTC cells. Of the 36 PTC tissue samples, 24 (66.7%) stained positive for ZNF703, while 6 (16.7%) of the normal thyroid tissue stained positive for ZNF703 ($P < 0.05$, Fig. 1c). These results suggest that ZNF703 is overexpressed in PTC.

Association of ZNF703 Protein Expression with Clinicopathological Features of PTC Patients

To evaluate the significance of ZNF703 expression to the progression of PTC, we determined whether ZNF703 protein expression may be associated with clinicopathological features of PTC patients. Our results indicate that ZNF703 expression was associated with tumor size, lymph node metastasis, and disease stage of PTC ($P < 0.05$, Table 1). No association was found between ZNF703 expression and age or gender ($P > 0.05$, Table 1). These results suggest that ZNF703 overexpression is associated with PTC progression.

Absence of Correlation between ZNF703 Expression and BRAF V600E Mutation

We also investigated BRAF V600E mutation in the same samples by IHC. The BRAF V600E mutation rate in our experiment was 58.3% (Fig. 1d). There was no significant correlation between the expression of ZNF703 and BRAF V600E mutation ($P < 0.05$, Table 2).

ZNF703-siRNA Reduces ZNF703 mRNA and Protein Levels in K1 Cells

To determine the function of ZNF703 in PTC, we used siRNA-mediated silencing to knock down the expression of ZNF703 in K1 human PTC cells. Transfection of ZNF703-siRNA reduced the expression of ZNF703 mRNA in K1 cells as compared with non-transfected cells (blank control) and cells transfected with a non-specific siRNA (negative control) ($P < 0.05$, Fig. 2a, 85.50 ± 1.93% reduction vs blank control). Similar results were obtained by Western blotting ($P < 0.05$, Fig. 2b, 65.0 ± 4.62% reduction vs blank control), though there were no statistical differences between the blank control and negative control groups ($P > 0.05$). These results verify that the ZNF703-siRNA can successfully inhibit ZNF703 mRNA and protein.

Effect of ZNF703-SiRNA on K1 Cell Proliferation

To assess the role of ZNF703 in the proliferation of PTC cells, we performed MTT assays. Our data show that the proliferation rate of K1 cells was significantly lower in the ZNF703-siRNA group than in the blank control and negative control groups over a 96-h time course starting at 24 h after transfection ($P < 0.01$, Fig. 3). There were no statistical differences between the blank control and negative control groups ($P > 0.05$). These results suggest that ZNF703 inhibition significantly suppresses the proliferation of a PTC cell line.

ZNF703 Silencing Promotes Apoptosis in K1 Cells

To further assess a potential role of ZNF703 in apoptosis, we performed flow cytometry (Annexin V/PI staining) and Hoechst 33342 staining. Our results suggest that K1 cells transfected with ZNF703-siRNA had a significantly higher apoptosis rate compared to the blank control and negative control ($P < 0.05$, Fig. 4). There were no statistical differences between the blank control and negative control groups ($P > 0.05$). These results suggest that ZNF703-siRNA induces cell apoptosis in K1 cells, which could explain its ability to suppress K1 cell proliferation.

ZNF703 Silencing Inhibits Invasion in K1 Cells

We also analyzed the effect of ZNF703-siRNA on regulation of K1 cell invasion using a Transwell invasion assay. Our data show that the number of cells crossing over the Matrigel after 72 h transfection were significantly reduced in the ZNF703-siRNA group as compared to the blank control and negative control groups ($P < 0.01$, Fig. 5). There were no statistical differences between the blank control and negative control

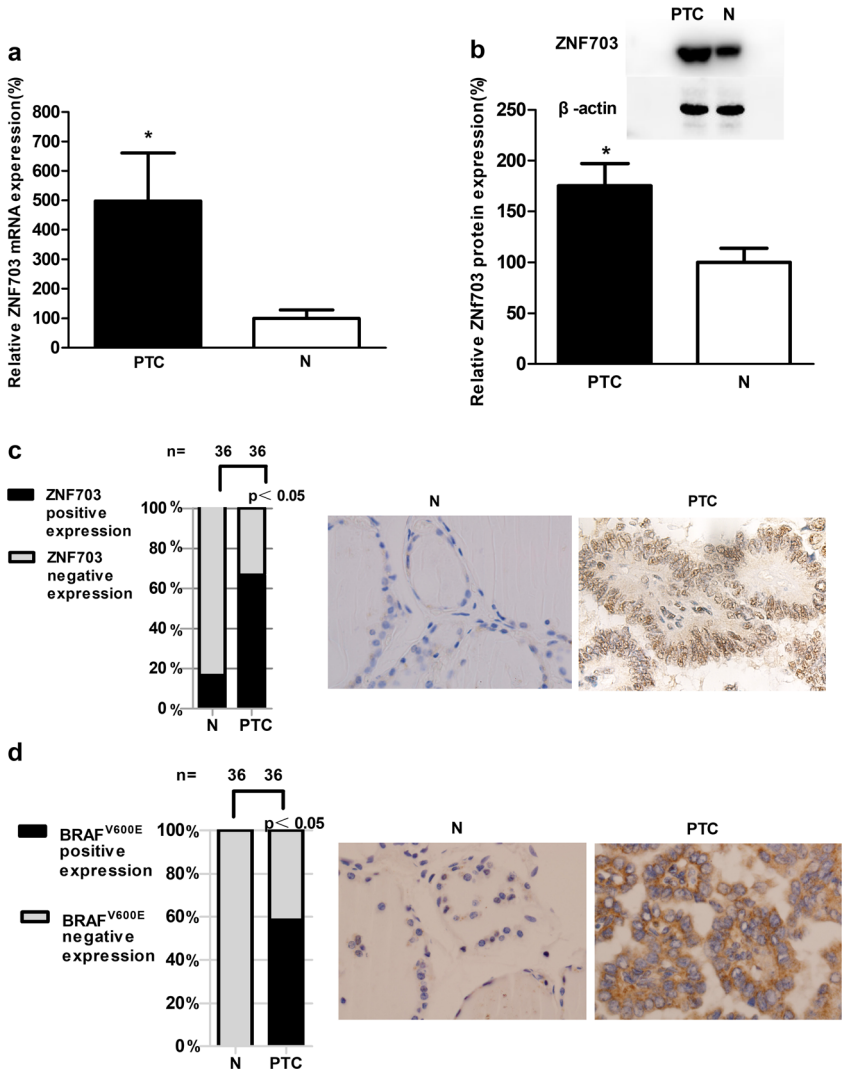

Fig. 1 ZNF703 and BRAF^{V600E} expression in PTC and normal thyroid tissues. **a** ZNF703 mRNA expression was analyzed by RT-PCR and was standardized to GAPDH mRNA. **b** ZNF703 protein expression was analyzed by western blotting and was standardized to β-actin protein. ZNF703 mRNA and protein levels were normalized to an average of 100% in normal tissue. Results represent the mean ± SD of tissues from 36 patients. **c, d** ZNF703 and BRAF^{V600E} protein expression was analyzed by immunohistochemical staining: quantification of the staining data (left) and representative staining from the tissues (right, * $P < 0.05$ versus normal tissue). *N* normal thyroid

groups ($P > 0.05$). These results indicate that ZNF703-siRNA also inhibits the invasion capacity of K1 cells.

ZNF703-siRNA Modulates the Downstream Regulatory Gene Expression in K1 Cells

To determine the impact of ZNF703 knockdown at the molecular level, we analyzed the effect of ZNF703-siRNA on the expression of genes that are known to be involved in tumor progression. ZNF703-siRNA significantly reduced the expression of E2F1 and MMP9 in K1 cells (42.8 ± 2.542% and 24.0 ± 1.93% reduction vs blank control; Fig. 6, $P < 0.05$), while the expression of p27 was significantly induced (124.5 ± 4.46% induction; Fig. 6, $P < 0.05$). There were no statistical differences between the blank control and negative control groups ($P > 0.05$). Furthermore, ZNF703-siRNA did not significantly change BRAF V600E protein levels. These results are consistent with the possibility that ZNF703 may function to promote cell proliferation and invasion through the modulation of E2F1, MMP9, and p27 expression.

Table 2 Relationship between ZNF703 and the mutation BRAF V600E expression

	BRAF V600E		r	*p*
	–	+		
ZNF703				
–	6	6		
+	9	15	0.12	0.487

Discussion

ZNF703 is a member of the NET family of zinc finger proteins, which is conserved from cnidarians to vertebrates and is linked to embryonic development. Members of this family (NocA, Nlz, Elbow, and TLP-1) are known to regulate gene expression, primarily as transcriptional repressors [26, 35]. NocA and

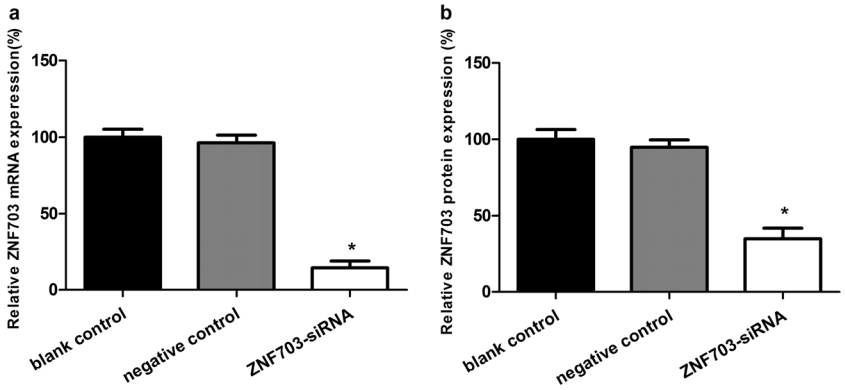

Fig. 2 Knockdown of ZNF703 in K1 cells. Reduced ZNF703 mRNA and protein levels in ZNF703-siRNA-transfected K1 cells were verified by RT-PCR and western blotting. **a** RT-PCR, expression of ZNF703 mRNA was standardized to GAPDH mRNA. **b** Western blotting, expression of ZNF703 protein is standardized to β-actin protein. The ZNF703 mRNA and protein levels are normalized to an average of 100% in blank control cells. * $P < 0.05$ versus blank control and negative control. Results represent the mean ± SD of three independent experiments

Elbow are found in Drosophila and are implicated in retina, trachea, wing and leg development [36, 37], while TLP-1 is found in *Caenorhabditis elegans* and is implicated in tail development [38]. Vertebrates have two NET family members, ZNF703 (NocA-like zinc finger 1/ Nlz1), and ZNF503 (NocA-like zinc finger 2/ Nlz2), each of which are implicated in developmental processes, such as brain patterning and limb formation [39, 40]. ZNF703 was first described in zebrafish [41] and is now known to be expressed in almost all human adult tissues [35]. Because ZNF703, like other NET proteins, lacks a nuclear localization signal, it interacts with other proteins that target it to the nucleus [30, 35]. Sircoulomb and coworkers determined that DCAF7, PHB2 and NCOR2 are co-factors of ZNF703 and facilitate its regulation of the expression of multiple genes [29]. Recently, ZNF703 was identified as an oncogene from an amplicon on 8p11.23 in estrogen receptor positive luminal B breast cancer [28, 29]. Furthermore, overexpression of ZNF703 has been associated with invasion and metastasis of breast cancer in mice [20]. Additionally, high levels of ZNF703 mRNA and protein have been detected in non-small cell lung cancer [42], gastric cancer [31], cholangiocarcinoma [32], oral squamous cell carcinoma [33]. However, little work has been done to explore ZNF703 mRNA and protein levels in PTC tumor samples and cell lines.

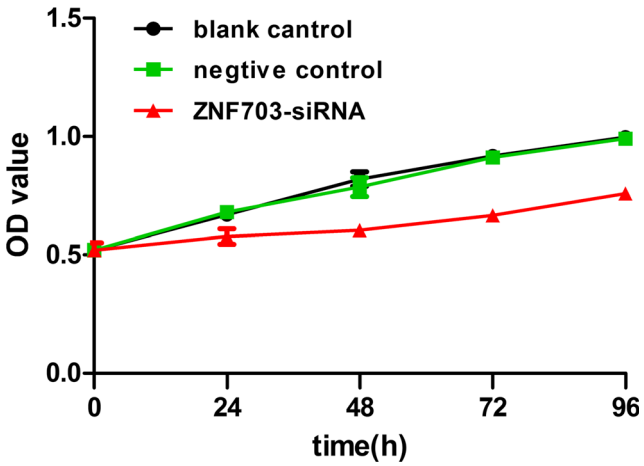

Fig. 3 ZNF703-siRNA inhibits K1 cell proliferation. Effects of ZNF703-siRNA on the cell proliferation were analyzed by MTT assay and measured as OD values. Results represent the mean ± SD of three independent experiments

In the present study, we performed RT-PCR and Western blotting of 36 PTC tissues sampled directly from patients. Our results demonstrate that ZNF703 mRNA and protein are highly expressed in PTC tissues compared with corresponding normal thyroid tissues. Immunohistochemical staining of 36 PTC tissues showed that ZNF703 is highly expressed in PTC tissues, with 66.7% of the tumors expressing ZNF703 compared with 16.7% of the normal thyroid tissue samples. These data verify that ZNF703 is overexpressed in the majority of PTC cases, suggesting that it may contribute to PTC development. ZNF703 expression in PTC was further demonstrated to correlate with tumor size, nodal metastasis, and clinical stage. The BRAF V600E mutation is the most common oncogenic mutation in PTC, and in the current study 21 (58.3%) of the PTCs had the BRAF V600E mutation. However, no significant correlation was detected between ZNF703 expression and BRAF V600E mutation as assessed by IHC, which suggests that ZNF703 may play a role in PTC independent of BRAF mutation. A larger study would be help to verify that the role of ZNF703 is independent of BRAF mutation. Regardless of the role of BRAF mutation, these results confirm the high expression of ZNF703 in PTC, which is consistent with the possibility that ZNF703 has transforming and tumorigenic properties in PTC.

To verify the oncogenic function of ZNF703 in PTC cells, we used ZNF703-siRNA to determine whether inhibition of ZNF703 in K1 cells affects processes related to tumor progression. Our results demonstrate that inhibition of ZNF703 results in the inhibition of cell proliferation and invasion, as well as the induction of apoptosis, which is consistent with

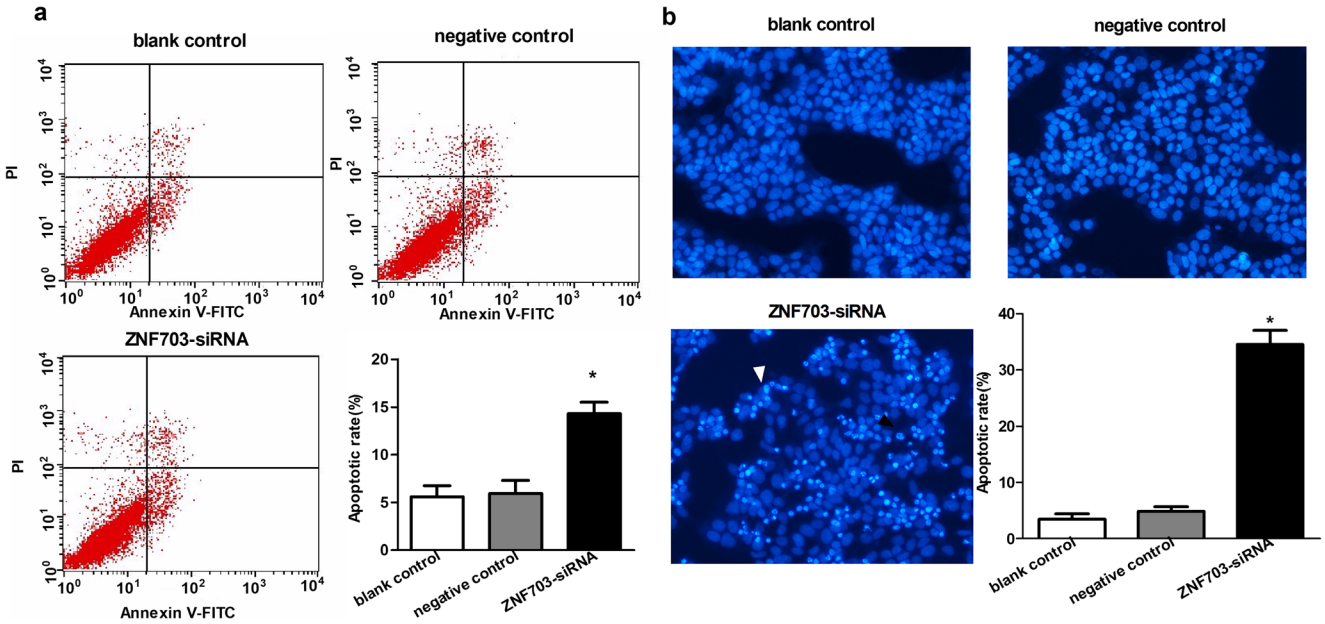

Fig. 4 ZNF703-siRNA induces K1 cell apoptosis. Induction of apoptosis in K1 cells transfected with ZNF703-siRNA was analyzed by flow cytometric analysis and Hoechst 33342 staining. **a** Flow cytometric analysis (Propidium iodide/ AnnexinV staining). **b** Hoechst 33342 staining. Quantification of the staining data represents the mean ± SD of three independent experiments. * $P < 0.05$ versus blank control and negative control

other reports in which breast cancer, gastric cancer and cholangiocarcinoma cells were suppressed through targeted inhibition of ZNF703 mRNA [30–32]. These results support the clinical findings and suggest that ZNF703 may provide a functional biomarker for PTC.

K1 cells have a BRAF V600E mutation [34]. In order to further explore whether ZNF703 is related to the expression of the BRAF V600E protein, we detected BRAF V600E levels after ZNF703 knockdown in K1 cells. However, there were no significant changes in the BRAF V600E protein levels after ZNF703 silencing, which is consistent with the immunohistochemistry results.

To elucidate the molecular mechanism of ZNF703 in enhancing the proliferation capability of PTC cells we analyzed

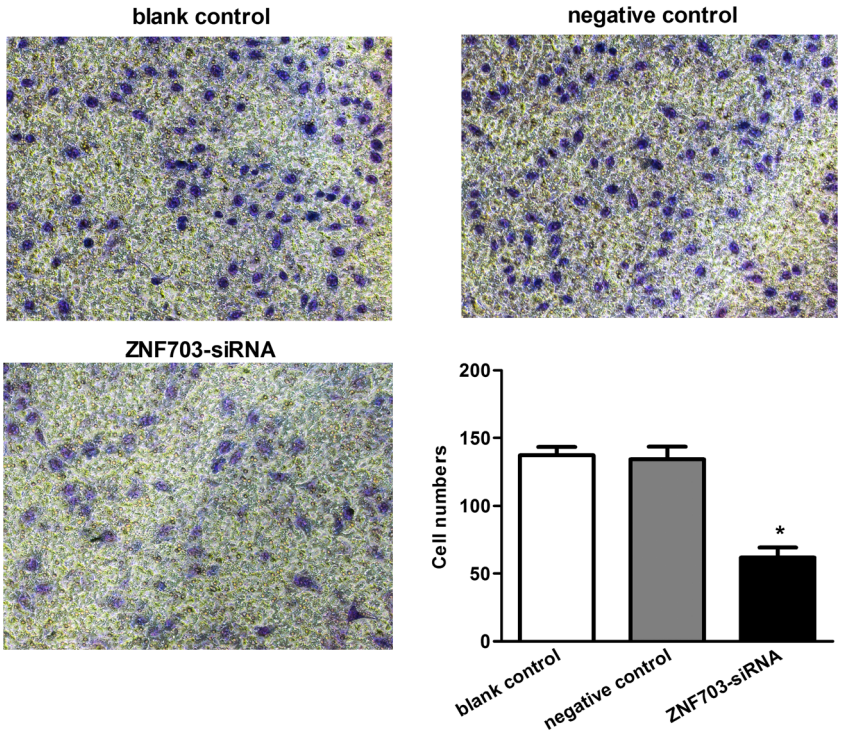

Fig. 5 ZNF703-siRNA inhibits K1 cell invasion. Inhibition of invasion in K1 cells transfected with ZNF703-siRNA was analyzed by Transwell analysis. Quantification of the staining data represents the mean ± SD of three independent experiments. * $P < 0.05$ versus blank control and negative control

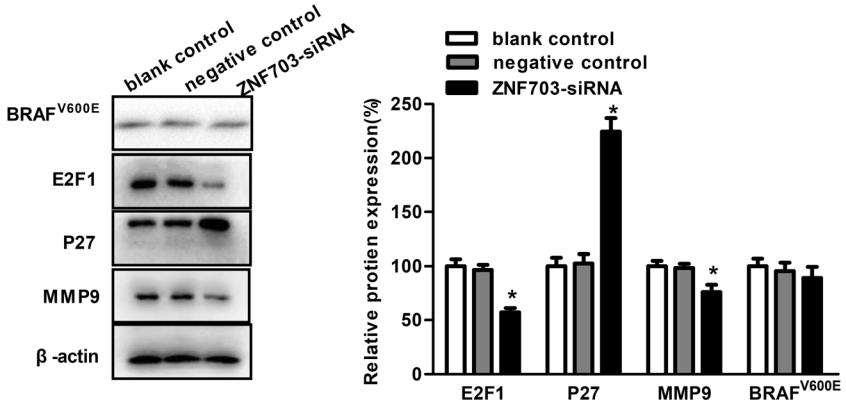

Fig. 6 ZNF703-siRNA modulates the expression of tumor-associated genes. Effects of ZNF703-siRNA in regulating gene expression in PTC cells. Representative image (left) shows downregulation of E2F1 and MMP9 and upregulation of p27 protein after transfection with ZNF703-siRNA; no significant changes in $BRAF^{V600E}$ protein expression were detected. Quantification of the relative protein levels represents the mean ± SD of three independent experiments. Values are normalized to an average of 100% in blank control cells. $*P < 0.05$ vs. blank control and negative control

the expression of its downstream regulatory genes E2F1 and p27. E2F1 is a transcription factor that induces cell cycle progression from G1 to S; and p27 is a check point inhibitor that blocks cell cycle progression through suppressing CyclinD/E-CDK2/4 activity. Rb is known to bind E2F1 and induce cell cycle arrest, while phosphorylation of Rb releases E2F1, leading to transcription of cell cycle genes (ex, c-myc, cyclinD1, and cyclinE1). E2F1 also induces proteolysis of p27, which allows the phosphorylation of Rb, resulting in increased E2F1 release, thus forming a positive feedback loop for mitotic entry [43–46]. Our results suggest that inhibition of ZNF703 in K1 cells downregulates E2F1 and upregulates p27, which is consistent with our cell proliferation studies. In support of these results, Fabrice and coworkers have shown that overexpression of ZNF703 may promote proliferation through the Rb/E2F1 protein network in MCF7 luminal cells [29]. Many studies have also suggested a role for E2F1 and p27 in tumorigenesis and aggressiveness [43, 47–49]. We propose, therefore, that the upregulation of E2F1 and downregulation of p27 by ZNF703 may explain the association of ZNF703 expression with the development and progression of PTC.

In addition to the Rb/E2F1 protein network, downregulation of p27 by ZNF703 could be the outcome of the interaction of ZNF703 within the ZNF703/DCAF7 complex [29]. DCAF7 is a CUL4/DDB1-interacting protein that can activate SKP2 (S-phase kinase associated protein2) to induce p27 and p53 proteolysis [50, 51]. P53 is mutated in 50% of human tumors, but K1 cells possess a wild-type p53 gene [52]. Thus, proteolysis of p53 by SKP2 could explain the induction of apoptosis after ZNF703 inhibition.

We also demonstrated that ZNF703 downregulation in K1 cells reduces the expression of MMP9, a member of the matrix metalloproteinase family that accelerates the aggressiveness of many tumors. Reports have shown that MMP9 is downstream of E2F1 and is involved in enhanced cellular invasion [53, 54]. Therefore, these results support our finding that tumor invasion is reduced in ZNF703-siRNA-expressing K1 cells. Tumor cell invasion is a precondition of metastasis. Therefore, these results also support our findings that ZNF703 expression in tissues from PTC patients correlates with lymph node metastasis.

Overall, we have demonstrated that ZNF703 is overexpressed in PTC and that its expression correlates with the disease state. Furthermore, silencing of ZNF703 affects the expression of E2F1, p27, and MMP9 to cause the inhibition of proliferation and invasion and the induction of apoptosis. This effect may not depend on the BRAF mutation. These results indicate that ZNF703 may be a key regulator of PTC development and progression. Additionally, ZNF703 may serve as a valuable target in PTC due to its link to E2F1.

Compliance with Ethical Standards

Conflict of Interest Statement All authors have no conflicts of interest.

Ethical Approval The Ethical Committee of Tangshan Gongren Hospital approved the study.

Informed Consent Informed consent was obtained from all patients.

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