Expression analysis of TRAF2- and NCK-interacting protein kinase (TNIK) and phosphorylated TNIK in papillary thyroid carcinoma

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Received January 11, 2023; Accepted May 10, 2023

DOI: 10.3892/ol.2023.13896

Abstract. The aim of the present study was to evaluate the expression of TRAF2- and NCK-interacting kinase (TNIK) and the levels of the active form of TNIK, phosphorylated (p)-TNIK, in papillary thyroid carcinoma (PTC), and to identify and compare the levels of TNIK and p-TNIK among PTC, benign thyroid tumors and normal tissues. The levels of TNIK and p-TNIK were examined by reverse transcription-quantitative (RT-q)PCR and immunohistochemical analysis (IHC) in PTC, benign thyroid tumors and normal tissues, and their association with clinicopathological features was evaluated. First, analysis of the Gene Expression Profiling Interactive Analysis and The Cancer Genome Atlas datasets suggested that the mRNA expression of TNIK was markedly increased in PTC tissues compared with that in normal tissues. RT-qPCR analyses then indicated that the relative mRNA expression of TNIK in PTC tissues was 4.47±6.16, which was significantly higher than that in adjacent tissues 2.57±5.83. The IHC results suggested that the levels of TNIK and p-TNIK in PTC tissues were markedly elevated compared with those in benign thyroid tumors and normal tissues. The levels of p-TNIK in patients with PTC were significantly associated with extrathyroidal extension (χ^2=4.199, P=0.040). Positive staining for TNIK was observed in 187 out of 202 (92.6%) cases in the cytoplasm, nucleus or cytomembrane of PTC cells. Among the 187 positive cases, cytoplasm expression was identified in 162 cases (86.6%), nuclear expression in 17 cases (9.1%) and cytomembrane expression in 8 cases (4.3%). Positive staining for p-TNIK was observed in 179 out of 202 (88.6%) cases in the nuclei, cytoplasm or cytomembrane of PTC cells. In the 179 p-TNIK-positive cases, localization in the nuclei plus cytoplasm was identified in 142 cases (79.3%), nuclear localization in 9 cases (5.0%), presence in the cytoplasm in 21 cases (11.7%) and cytomembrane localization in 7 cases (3.9%). Both TNIK and p-TNIK were upregulated in PTC tissues and p-TNIK was significantly associated with extrathyroidal extension. It may act as a crucial oncogene to participate in PTC carcinogenesis and progression.

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Key words: TRAF2- and NCK-interacting kinase, p-TNIK, papillary thyroid carcinoma

Introduction

Thyroid carcinoma (TC) is the most frequent malignant neoplasm of the endocrine system and its incidence rate has been steadily increasing with an annual growth rate of 4.5-6.6% across the world (1). In 2018 alone, according to the Surveillance, Epidemiology and End Results Program from the National Cancer Institute, nearly 54,000 new cases of TC were registered, accounting for 3.1% of all new cases of cancer during this year so far (2).

Papillary TC (PTC) is the most common type of thyroid malignancy, accounting for >80% of all thyroid cancers (3), and an even higher proportion of 95.1% was estimated in the Chinese population (4). Over the past decade, the diagnosis level of PTC has markedly improved due to the wide use of fine-needle aspiration cytology (FNAC) together with the detection of B-Raf proto-oncogene, serine/threonine kinase (BRAF)^{V600E} mutation in clinical practice (5). FNAC is routinely used as the main tool in the preoperative evaluation of thyroid nodules. However, ~15-30% of FNA specimens were reported to give inconclusive results, which are read as 'indeterminate' or 'suspicious malignancy', offering a challenge in terms of interpretation and clinical management (6). As for the BRAF^{V600E} mutation, despite a high specificity (1.00, 95% CI: 0.98-1.00) for PTC, BRAF^{V600E} mutation has a low overall sensitivity [(0.40, 95% CI: 0.32-0.48) or (0.60, 95% CI: 0.556-0.634)], limiting its diagnostic value as a single screening test (7,8). Although most patients with PTC have an excellent prognosis (9), the prevalence of PTC still raises concern due to a recurrence rate of almost 30% and cause-specific mortality of 8.6% for a three-decade period (10). Therefore, it becomes particularly urgent and necessary to screen novel tumor markers and new therapeutic targets for PTC.

TRAF2- and NCK-interacting protein kinase (TNIK) is one of the germinal center kinase family members, localized in chromosome region 3q26, where gene amplification often occurs in various cancers (11,12). Accumulating evidence suggested that TNIK, as a protein interacting with transcription factor 4 (TCF4) (13,14), is involved in extensive biochemical pathways, such as the JNK, Wnt/β-catenin or PI3K/Akt pathways, which are shared between embryogenesis and tumorigenesis (11,13,15). TNIK is essential for the transactivation of Wnt signal target genes (13,16) and its expression is associated with poor prognosis of patients with hepatocellular (17), colorectal (18) and pancreatic (19) cancers. Amplification of the TNIK gene is detectable in 7% of gastric cancers (11) and 50% of lung carcinomas (12), and TNIK is reportedly one of several putative driver oncogenes (20). Furthermore, TNIK-targeted treatments were revealed to be potential therapies for colorectal cancer (21), synovial sarcoma (22), lung cancer (23), osteosarcoma (24), prostate cancer (25) and breast cancer (26). Therefore, in the present study, the expression of TNIK and its activated form, phosphorylated (p)-TNIK, were preliminary investigated and compared among PTC samples, benign thyroid tumors and normal thyroid tissues, and the results revealed that both TNIK and p-TNIK were upregulated in PTC compared to benign tumors and normal tissues. The expression of p-TNIK was positively associated with extrathyroidal extension in patients with PTC.

Patients and methods

Public datasets. The expression pattern of TNIK in multiple cancer types was downloaded from the Gene Expression Profiling Interactive Analysis (GEPIA) dataset (http://gepia.cancer-pku.cn/). The expression of TNIK in PTC tissues compared with that in normal tissues was downloaded from the public The Cancer Genome Atlas (TCGA) dataset (https://ualcan.path.uab.edu/analysis.html).

Patients. Patients who were diagnosed with primary PTC and underwent subtotal thyroidectomy or radical resection at the Department of Otolaryngology Head and Neck Surgery at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between June 2021 and June 2022 were enrolled in the present study. Samples from patients who suffered from benign thyroid tumors and underwent subtotal thyroidectomy during the same period were collected as controls. Furthermore, isthmuses of the thyroid gland of patients who underwent tracheotomy, without benign or malignant thyroid tumors, between October 2015 and June 2022 were considered normal thyroid tissues and included in the study.

The use of tissues for this study was approved by the Research Ethics Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China), and conformed to all relevant ethical regulations for human research subjects in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants before any clinical samples were collected. The excluded patients were breast-feeding patients, those with other benign or malignant tumors, or severe cardiovascular or renal diseases.

A total of 202 patients with PTC were assigned to the mRNA analysis by reverse transcription-quantitative (RT-q) PCR to compare the relative mRNA expression of TNIK in PTC tissue and their matched adjacent tissue. Furthermore, 202 PTC tissues, 150 PTC-adjacent tissues, 100 benign thyroid tumor tissues accompanied by PTC (termed as benign tumor A), 100 benign thyroid tumors not accompanied by PTC (termed as benign tumor B) and 100 normal thyroid tissues were subjected to immunohistochemistry (IHC) to detect the TNIK and p-TNIK protein levels. For RT-qPCR analysis, a strip of tumor tissue (without any adjacent tissue as much as possible) and corresponding adjacent tissues (at least 1 cm apart from the PTC tissue) were collected. Immediately after excision, the tissue samples were stored in M5 HiPer RNA stay (Mei5bio) and placed in a -80˚C refrigerator. The paraffin-embedded specimens for IHC analysis were produced by the Pathology Department of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China).

RNA extraction and RT-qPCR. RNA was extracted from PTC tissues and corresponding adjacent tissues using the Eastep® SuperTotal RNA Extraction Kit (Promega Corp.) according to the manufacturer's protocol. RNA concentration and quality were assessed using a NanoDrop® One spectrophotometer (Thermo Fisher Scientific, Inc.). The GoScript™ Reverse Transcription Mix (Promega Corp.) was used to generate cDNA from RNA according to the manufacturer's instructions. The amplification reaction was performed using GoTaq® qPCR Master Mix (Promega Corp.) according to the manufacturer's protocol. The qPCR procedure was as follows: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. qPCR was performed using a QuantStudio DX Real-Time PCR system (Thermo Fisher Scientific, Inc.) with each reaction run in triplicate. The relative target gene mRNA expression was determined using the comparative Ct method (27), with GAPDH as the endogenous control. The primer sequences were as follows: TNIK forward, 5'-GGTAGAAGAACGGTCAAGGCTCAAC-3' and reverse, 5'-GGCTGAACTCCACTAATGCTGAAGG-3'; GAPDH forward, 5'-AATCCCATCACCATCTTCCA-3' and reverse, 5'-TGGACTCCACGACGTACTCA-3'.

Immunohistochemistry (IHC). According to the manufacturer's instructions, IHC staining was performed using the UltraSensitive TMSP (Rabbit) IHC Kit (cat. no. PV-9001; OriGene Technologies, Inc.). First, the antigens were retrieved by autoclaving them at 121˚C for 2 min in pH 6.0 citrate buffer (Fuzhou Maixin Biotech. Co., Ltd.). The slides were then incubated in a solution of endogenous peroxidase blocker (OriGene Technologies, Inc.) for 15 min at room temperature in order to quench endogenous peroxidase activity. Thereafter, the slides were incubated with a rabbit polyclonal antibody against TNIK at 1:150 dilution (cat. no. ab224252; Abcam) or p-TNIK (Ser764) at 1:300 dilution (cat. no. bs-5598R; BIOSS) overnight at 4˚C. Next, the slides were incubated with reaction strengthening fluid (OriGene Technologies, Inc.) for 20 min at 37˚C, followed by incubation with enhanced enzyme-labeled goat anti-rabbit IgG polymer (cat. no. PV-9001; OriGene Technologies, Inc.) at 37˚C for 20 min. Color development was performed with DAB (OriGene Technologies, Inc.) for 5 min at room temperature. The slides were counterstained with Harris hematoxylin, after which they were dehydrated

using a series of increasing alcohol concentrations, and finally mounted with cover slips.

IHC staining was evaluated according to a previously reported scoring method (28). All slides were scored by three experienced pathologists blinded to the clinical data. The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strongly positive). The staining extensity was scored as 0 (0-25% of the tumor cells stained), 1 (26-50%), 2 (51-75%) or 3 (76-100%). The sum of the intensity and extensity scores, potentially ranging from 0 to 6, was calculated, and the average of five fields (magnification, x400) was used to determine the TNIK and p-TNIK staining score for each patient. Expression was classified as low when staining scores were ≤2 and cases were defined as high when staining scores were ≥3. Cases were defined as positive when the staining intensity was more than weakly positive (weak + moderate + strong) and >10% of tumor cells were positive.

Statistical analysis. All statistical analyses were performed using SPSS version 21.0 software for Windows (IBM Corp.). Measurement data were expressed as the mean ± standard deviation and analyzed by Student's t-test. One-way ANOVA was performed for multiple comparisons and post-hoc testing was used to compare two groups among multiple comparisons. Matched tissue comparisons were performed with a paired t-test, while comparisons between non-matched samples were performed by two-samples t-tests. The χ^2 test was used to evaluate the association of expression with the clinicopathological parameters. Fisher's exact test was performed to replace the χ^2 test in cases with low numbers. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of TNIK and p-TNIK is upregulated in PTC tissues. To explore the potential role of TNIK involved in PTC tumorigenesis, the expression pattern of TNIK in multiple cancer types from the GEPIA dataset was first analyzed. The results indicated that TNIK was significantly upregulated in TC (THCA) compared to normal tissues (Fig. 1A). Subsequently, the public TCGA dataset was analyzed and it was also found that the mRNA expression of TNIK was markedly increased in PTC tissues compared with that in normal tissues (Fig. 1B). To further investigate the relative expression of TNIK in PTC tissues and matched adjacent tissues, the expression of TNIK in 202 paired PTC and adjacent tissues was comparatively analyzed using RT-qPCR. The results indicated that the relative mRNA expression of TNIK in PTC tissues was 4.47±6.16, which was markedly higher compared with the relative expression of mRNA in adjacent tissues 2.57±5.83 (P=0.0015) (Fig. 1C). Next, IHC analysis was performed to detect the protein levels of TNIK and p-TNIK in PTC tissues, PTC adjacent tissues, benign thyroid tumors and normal tissues. A total of 202 paired samples of fresh PTC and adjacent tissues were obtained. However, not every adjacent tissue block was sufficient to be made into a wax block, and in the subsequent IHC analysis, 202 PTC and 150 adjacent tissue wax blocks were available for the analysis of TNIK and p-TNIK. The IHC results indicated that the level of TNIK and p-TNIK in PTC tissues was markedly elevated compared with that in benign thyroid tumors A, benign thyroid tumors B, adjacent tissue and normal tissues (Fig. 1D and E). There was no significant difference in TNIK and p-TNIK between benign thyroid tumors A and benign thyroid tumors B, suggesting that the elevated TNIK and p-TNIK levels in PTC tissues did not implicate the adjacent benign thyroid tumors. Similarly, there was no significant difference between PTC adjacent tissue and normal tissues, indicating that the upregulation of TNIK and p-TNIK in PTC tissue did not include the adjacent tissue.

Association of TNIK and p-TNIK levels with clinicopathological characteristics of patients with PTC. To determine the clinical significance of TNIK and p-TNIK in PTC, the relationship between TNIK or p-TNIK levels and clinicopathological parameters was analyzed. The expression of TNIK in patients with PTC was not associated with gender, age, tumor size, multifocality, extrathyroidal extension, lymph node (LN) metastasis or TNM stage ($P>0.05$). p-TNIK expression was more frequently observed in the extrathyroidal extension group ($\chi^2=4.199$, $P=0.040$), while there was no association between p-TNIK and gender, age, tumor size, multifocality, LN metastasis or TNM stage ($P>0.05$; Table I).

Levels of TNIK and p-TNIK in PTC. Based on the data above, there was no significant difference in the levels of TNIK and p-TNIK between benign thyroid tumors A and benign thyroid tumors B, as well as between PTC adjacent tissue and normal tissues. In the following analysis, benign thyroid tumors B were selected as benign tumors, and in addition, normal tissues were used for further investigation. TNIK-positive staining was observed in 187 out of 202 (92.6%) cases in the cytoplasm, nuclei or cytomembrane of PTC cells. In the 187 positive cases, cytoplasm expression was identified in 162 cases (86.6%), nuclear expression in 17 cases (9.1%) and cytomembrane expression in 8 cases (4.3%) (Table II, Fig. 2). There was no significant difference in expression location distribution among PTC tissue, benign thyroid tumors and normal tissue (Table II).

p-TNIK-positive staining was observed in 179 out of 202 (88.6%) cases in the nuclei, cytoplasm or cytomembrane of PTC cells. In the 179 p-TNIK-positive cases, nuclear plus cytoplasm expression was identified in 142 cases (79.3%), nuclear expression in 9 cases (5.0%), cytoplasm expression in 21 cases (11.7%) and cytomembrane expression in 7 cases (3.9%) (Table III, Fig. 3). Specifically, the most positive p-TNIK expression pattern was strong staining in nuclei plus weak or strong staining in the cytoplasm. In comparison, in benign thyroid tumors and normal tissue, the main positive expression was located in the nuclei (Table III).

Comparison of sensitivity and specificity among $BRAF^{V600E}$ mutation, TNIK expression and p-TNIK levels in PTC diagnosis. The sensitivity and specificity of $BRAF^{V600E}$ mutation (routinely detected by the Molecular Cell Diagnostic Center of our institution) in the patients enrolled in the present study were 68.6 and 89.7%, respectively (Table IV), which indicated a higher sensitivity and a lower specificity compared to prior studies (7,8). As presented in Tables V and VI, the sensitivity and specificity of TNIK and p-TNIK in the diagnosis of PTC were 92.6 and 11.0%, respectively, as well as 88.6 and 19.0%,

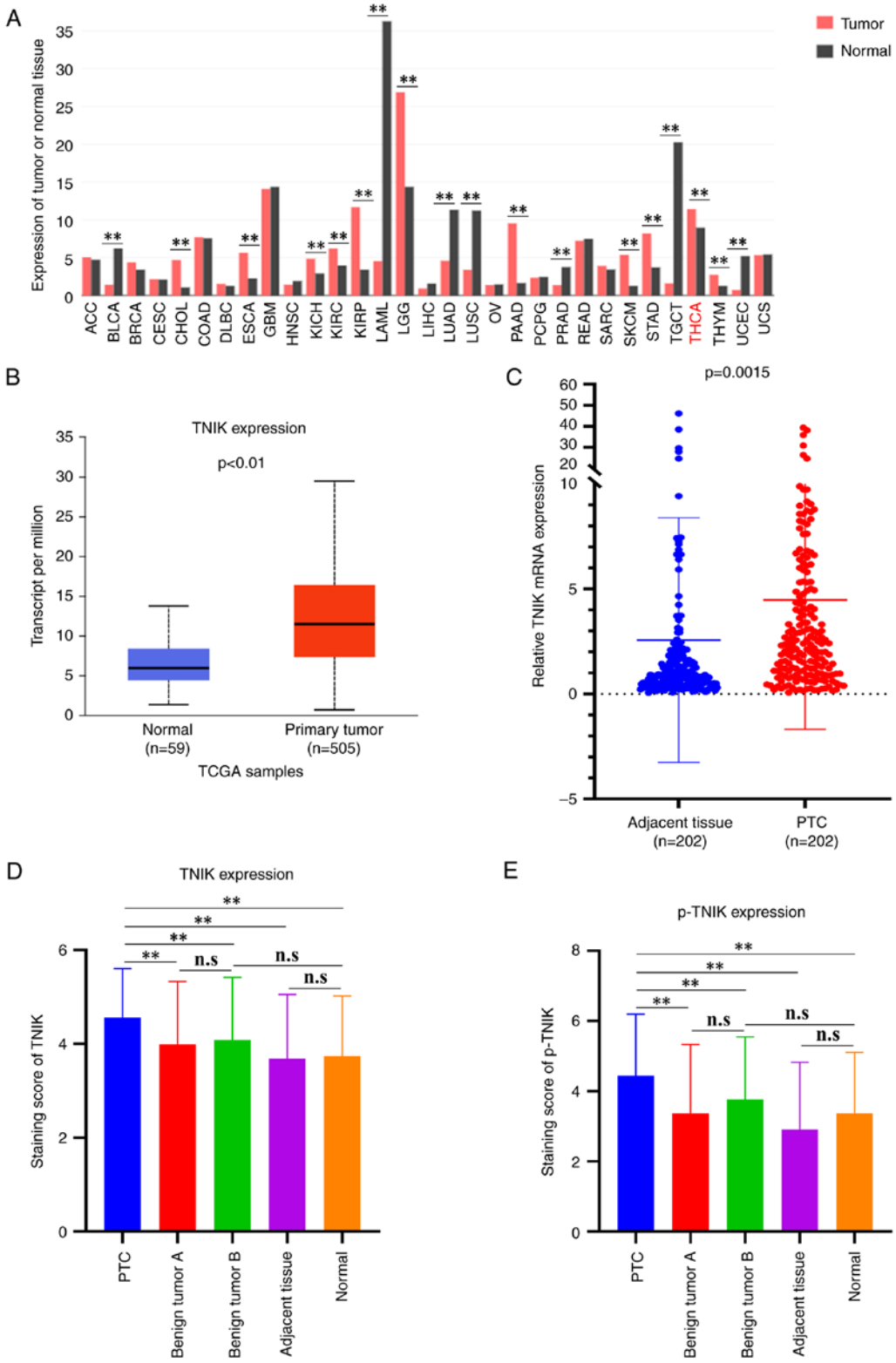

Figure 1. TNIK and p-TNIK are upregulated in PTC tissue. (A) The expression of TNIK mRNA in diverse cancers was determined from GEPIA dataset. (B) The expression of TNIK mRNA in PTC was determined from the TCGA dataset. (C) The mRNA expression of TNIK was detected by reverse transcription-quantitative PCR in 202 pairs of PTC tissues and matched adjacent tissues. (D) The protein expression of TNIK was analyzed by IHC in PTC tissues, benign thyroid tumors, adjacent tissues and normal tissues. (E) The protein levels of p-TNIK were analyzed by IHC in PTC tissues, benign thyroid tumors, adjacent tissues and normal tissues. **P<0.01. n.s., no significance; p-TNIK, phosphorylated TRAF2- and NCK-interacting kinase; IHC, immunohistochemistry; TCGA, The Cancer Genome Atlas; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; PTC, papillary thyroid carcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma.

Table I. Association of TNIK and p-TNIK levels with clinicopathological characteristics of patients with papillary thyroid carcinoma.

		TNIK			p-TNIK			
Characteristic	n	High (n=168)	Low (n=34)	P-value	n	High (n=172)	Low (n=30)	P-value
Gender				0.388				0.056
Male	60	52 (86.7)	8 (13.3)		56	52 (92.9)	4 (7.1)	
Female	142	116 (81.7)	26 (18.3)		146	120 (82.2)	26 (17.8)	
Age, years				0.083				0.579
<55	154	132 (85.7)	22 (14.3)		168	142 (84.5)	26 (15.5)	
≥55	48	36 (75.0)	12 (25.0)		34	30 (88.2)	4 (11.8)	
Tumor size, cm				0.846				0.380
<1	110	92 (83.6)	18 (16.4)		120	100 (83.3)	20 (16.7)	
≥1	92	76 (82.6)	16 (17.4)		82	72 (87.8)	10 (12.2)	
Multifocality				0.092				0.788
Single	142	114 (80.3)	28 (19.7)		144	122 (84.7)	22 (15.3)	
Multifocal	60	54 (90.0)	6 (10.0)		58	50 (86.2)	8 (13.8)	
Extrathyroidal extension				0.381				0.040
Positive	70	56 (80)	14 (20)		74	68 (91.9)	6 (8.1)	
Negative	132	112 (84.8)	20 (15.2)		128	104 (81.3)	24 (18.7)	
LN metastasis				0.562				0.267
Positive	86	70 (81.4)	16 (18.6)		86	76 (88.4)	10 (11.6)	
Negative	116	98 (84.5)	18 (15.5)		116	96 (82.8)	20 (17.2)	
TNM stage				0.193				1.000
I	184	155 (84.2)	29 (15.8)		186	158 (84.9)	28 (15.1)	
II/III/IV	18	13 (72.2)	5 (27.8)		16	14 (87.5)	2 (12.5)	

The χ^2 test was used for comparing groups between high and low TNIK or p-TNIK expression. p-TNIK, phosphorylated TRAF2- and NCK-interacting kinase; LN, lymph node.

respectively, affirming that both TNIK and p-TNIK have high sensitivity and poor specificity. In spite of the desirable sensitivity compared to $BRAF^{V600E}$ mutation, in view of the unsatisfactory specificity, TNIK and p-TNIK may be regarded as oncogenes, but not be used as indicators for PTC diagnosis.

Discussion

The present study was the first, to the best of our knowledge, to determine the protein levels of TNIK and p-TNIK in PTC clinical tissue samples. The results indicated that TNIK and p-TNIK were significantly elevated in PTC tissues compared to benign thyroid tumors and normal tissues, and p-TNIK was significantly associated with extrathyroidal extension, while the expression of TNIK did not exhibit any association with any of the clinicopathological parameters of the patients with PTC, which explained that p-TNIK, as an active form of TNIK, may have a crucial role in regulating transcriptional activity in PTC. IHC analysis then indicated that the expression of TNIK was mainly located in the cytoplasm, while the location of p-TNIK was in the nuclei and cytoplasm. Strikingly, to sum up, the present study suggested that p-TNIK/TNIK may be considered an oncogene to participate in the carcinogenesis of PTC.

Multifocality in PTC is common and has been considered a significant risk factor for disease progression and risk of recurrence in PTC (29). Therefore, in the present study, to verify whether benign thyroid tumors are implicated in the accompanied PTC, the relative expression of TNIK and levels of p-TNIK were detected and compared in benign thyroid tumors accompanied by PTC and benign thyroid tumors not accompanied by PTC. The results indicated that there was no significant difference in TNIK and p-TNIK expression between benign thyroid tumors from the two different groups, which suggested that the elevated TNIK and p-TNIK expression in PTC tissue did not include the adjacent benign thyroid tumors. Similarly, there was no significant difference between PTC adjacent tissue and normal tissues, indicating that the upregulated TNIK and p-TNIK in PTC tissues did not extend to the adjacent tissues.

The phosphorylation and dephosphorylation of proteins on serine residues are essential for regulating a broad range of cellular functions in eukaryotes, including cell division, homeostasis and apoptosis (30,31). The serine 764 (S764) residue of human TNIK has been identified as a phosphorylation site by liquid chromatography tandem mass spectrometry-based random sequencing of protein kinases (32). P-TNIK then translocates into the nucleus

Table II. Positive expression percentage of TNIK in cytoplasm, nuclei and cytomembrane of different samples.

		TNIK expression			
Sample type	n	Cytoplasm	Nuclear	Cytomembrane	P-value
PTC	187	162 (86.6)	17 (9.1)	8 (4.3)	0.883
Benign tumor	89	76 (85.4)	9 (10.1)	4 (4.5)	
Normal	83	75 (90.4)	6 (7.2)	2 (2.4)	

The χ^2 test was used for comparing TNIK expression in cytoplasm, nuclei and cytomembrane. TNIK, TRAF2- and NCK-interacting kinase; PTC, papillary thyroid carcinoma.

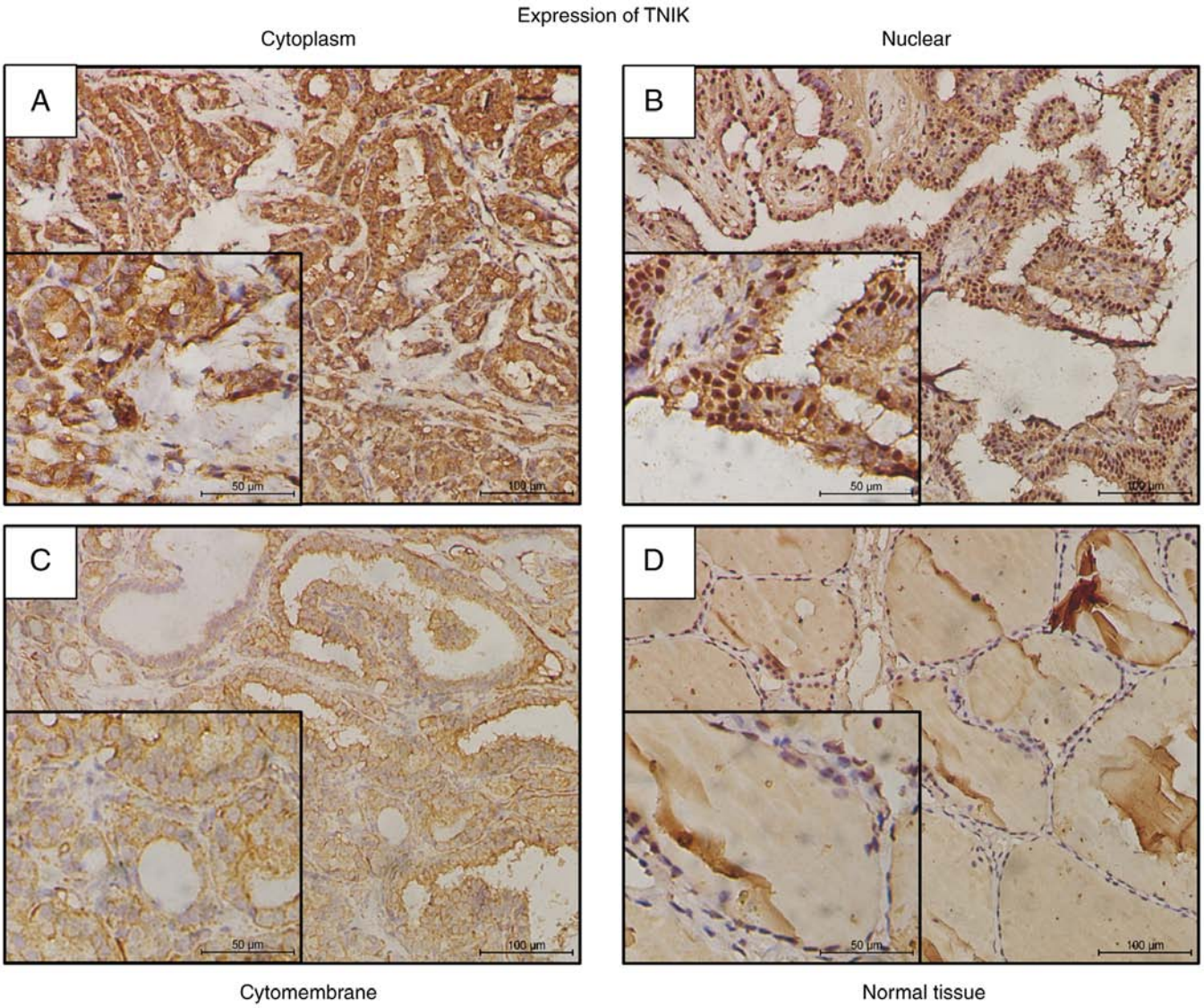

Figure 2. Representative immunohistochemical images for the protein expression of TNIK in PTC and normal tissues. (A-C) TNIK staining indicating (A) cytoplasm expression, (B) nuclear expression and (C) cytomembrane expression in PTC. (D) Staining for TNIK in normal tissues (scale bars, 100 μm; inner figures: Magnification, x400; scale bars, 50 μm). TNIK, TRAF2- and NCK-interacting kinase; PTC, papillary thyroid carcinoma.

and augments the transcriptional activity of TCF4. A study reported that TNIK-positive staining was detected in 92.7% of hepatocellular carcinomas in the cytoplasm and p-TNIK expression was identified in the cytoplasm of 55.6% and nuclei of 7.9% of samples (17). A study on colorectal cancer suggested that TNIK protein was observed in the cytoplasm of cancer cells (18) and TNIK protein was distributed along the filamentous cytoskeleton, whereas p-TNIK was detected mainly in the nuclei and colocalized with the TCF4/β-catenin complex (16). This was almost coincident

Table III. Percentage of positivity for p-TNIK in nuclei, cytoplasm and cytomembrane of different samples.

		Positivity for p-TNIK				
Sample type	n	Nuclear plus cytoplasm	Nuclear	Cytoplasm	Cytomembrane	P-value
PTC	179	142 (79.3)	9 (5.0)	21 (11.8)	7 (3.9)	
Benign tumor	81	0 (0)	69 (85.2)	7 (8.6)	5 (6.2)	<0.001
Normal	85	0 (0)	73 (85.9)	9 (10.6)	3 (3.5)	

The χ^2 test was used for comparing p-TNIK expression in cytoplasm, nuclei and cytomembrane. p-TNIK, phosphorylated TRAF2- and NCK-interacting kinase; PTC, papillary thyroid carcinoma.

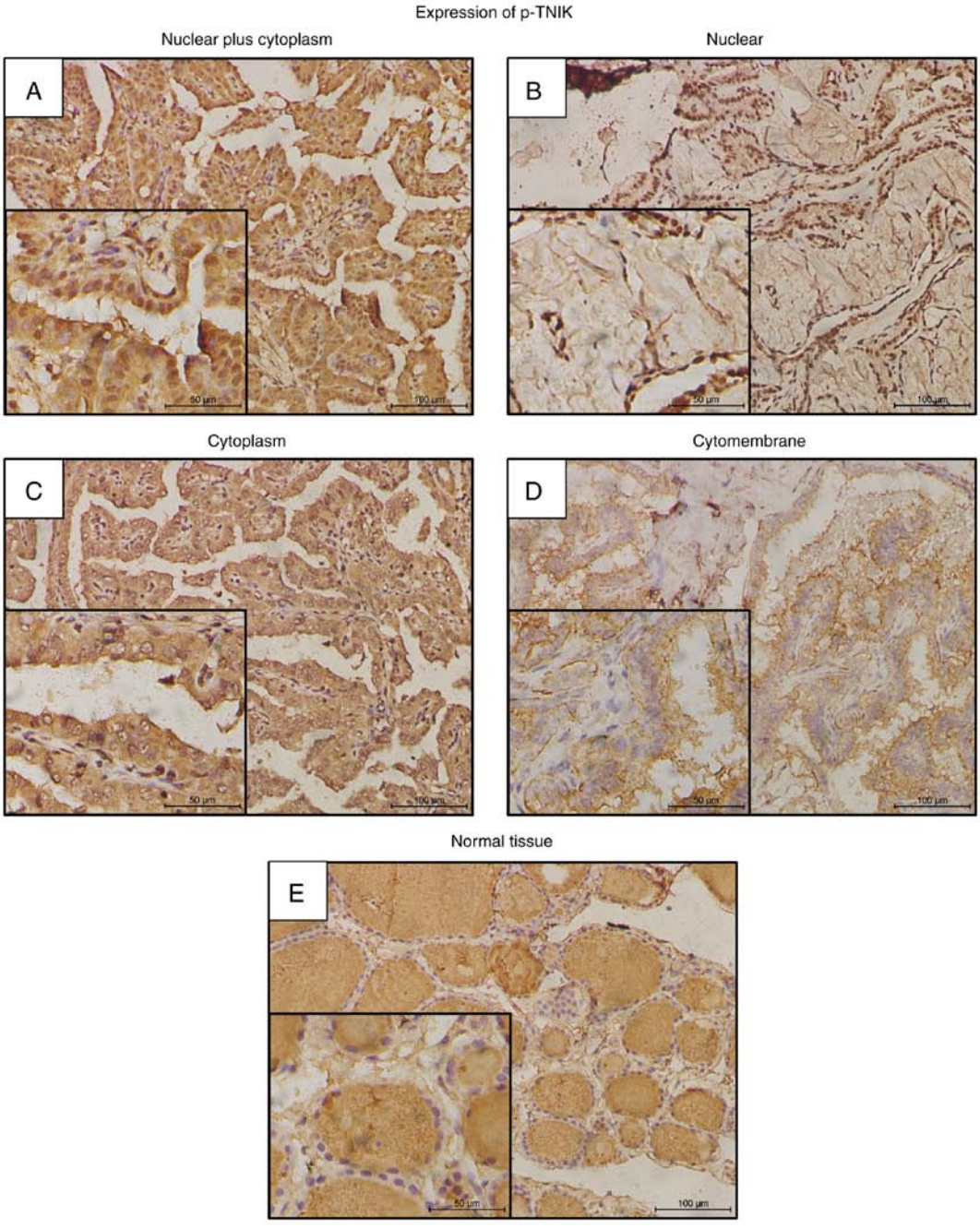

Figure 3. Representative immunohistochemical images for the protein levels of p-TNIK in PTC and normal tissues. (A-D) p-TNIK staining of (A) nuclear plus cytoplasm expression, (B) nuclear expression, (C) cytoplasm expression and (D) cytomembrane expression in PTC. (E) Staining for p-TNIK in normal tissues (scale bars, 100 μm; inner figures: Magnification, x400; scale bars, 50 μm). p-TNIK, phosphorylated TRAF2- and NCK-interacting kinase; PTC, papillary thyroid carcinoma.

Table IV. Specificity and sensitivity of $BRAF^{V600E}$ mutation in PTC diagnosis.

	Pathologic diagnosis of PTC	
$BRAF^{V600E}$	+	-
+	107 (68.6) (sensitivity)	7 (10.3)
-	49 (31.4)	61 (89.7) (specificity)

Values are expressed as n (%). PTC, papillary thyroid carcinoma; BRAF, B-Raf proto-oncogene, serine/threonine kinase.

Table V. Specificity and sensitivity of TNIK expression in PTC diagnosis.

	Pathologic diagnosis of PTC	
TNIK expression	+	-
+	187 (92.6) (sensitivity)	89 (89.0)
-	15 (7.4)	11 (11.0) (specificity)

Values are expressed as n (%). PTC, papillary thyroid carcinoma; TNIK, TRAF2- and NCK-interacting kinase.

Table VI. Specificity and sensitivity of p-TNIK positivity in PTC diagnosis.

	Pathologic diagnosis of PTC	
p-TNIK status	+	-
+	179 (88.6) (sensitivity)	81 (81.0)
-	23 (11.4)	19 (19.0) (specificity)

Values are expressed as n (%). PTC, papillary thyroid carcinoma; p-TNIK, phosphorylated TRAF2- and NCK-interacting kinase.

with our finding that TNIK-positive staining was mainly located in the cytoplasm and p-TNIK-positive staining was present in the nuclei plus cytoplasm. A noteworthy finding was that the most positive p-TNIK expression pattern in PTC was strong staining in nuclear plus weak or strong staining in the cytoplasm. However, in benign thyroid tumors and normal tissue, the positive expression of p-TNIK was mainly located in the nuclei. As is well known, human cancers are a heterogeneous disease and multiple cancer genes consist of all genetic alterations that modify the normal DNA/mRNA sequences triggering a cataract of molecular reactions (33). Therefore, it was speculated that in the tumorigenesis of PTC, one or more genes hinder the transfer of p-TNIK from the cytoplasm to the nucleus, and this key finding may help uncover the mechanism of PTC. Furthermore, at least, in the present study, this different location of p-TNIK may help distinguish PTC from benign tumors.

Emerging evidence suggested that TNIK as an oncogene is involved in the progression of gastric cancer, lung cancer, pancreatic cancer, colorectal cancer, synovial sarcoma, osteosarcoma, prostate cancer and breast cancer (11,12,19,21-26), while p-TNIK expression was observed to be increased in hepatocellular carcinoma and ERG-positive prostate cancer and associated with poor prognosis (17,25). Based on this, it was speculated that TNIK may be an oncogene that participates in PTC tumorigenesis. In the current first study of TNIK expression in PTC, both TNIK and p-TNIK were found to be upregulated in PTC and p-TNIK was more frequently observed in extrathyroidal extension, while the expression of TNIK did not exhibit any association with any clinicopathological parameters of patients with PTC. When TNIK is activated, the resulting p-TNIK is upregulated and the tumor cells detach and disseminate, leading to metastasis (17). It was indicated that high levels of p-TNIK were coincident with tumor progression, which suggests that p-TNIK may serve as a tumor activator in PTC. Taken together, the present study suggests that high levels of p-TNIK may function as an oncogene and have an important role in the progression of PTC.

$BRAF^{V600E}$ mutation, as one of the most common mutations, is frequently present in thyroid cancer (34,35). Of note, the $BRAF^{V600E}$ mutation in thyroid cancer occurs in ~50% of PTC and PTC-derived anaplastic TC cases, but rarely occurs in follicular TC or other types of thyroid tumor (36). The data of the present study indicated a higher sensitivity and a lower specificity of $BRAF^{V600E}$ mutation compared to prior studies, which perhaps resulted from the limited sample number. In spite of the desirable sensitivity compared to $BRAF^{V600E}$ mutation, TNIK and p-TNIK were impractical to be adopted as diagnostic indicators for PTC due to the poor specificity. However, the present work provides important insight into TNIK or p-TNIK serving as a novel biomarker, and the distinct differences in expression location of p-TNIK between PTC and benign tumor may contribute to PTC diagnosis.

Besides as an oncogene, another research focus on TNIK is its utility as a target molecule for anti-cancer treatment. TNIK has recently been considered a first-in-class anti-cancer target molecule to regulate the Wnt signaling pathway. Previous studies have proved that the small-molecule TNIK inhibitor NCB-0846 suppressed tumorigenesis, epithelial-to-mesenchymal transition, cell viability, colony formation and apoptotic cell death *in vitro* and induced regression of xenografts or abolished cancer metastasis in *in vivo* models (21-25). 108600, as a novel TNIK inhibitor, was confirmed to suppress the growth and colony- and mammosphere-forming capacity of breast cancer stem cell-like cells, induce apoptosis and overcome chemotherapy resistance in mice bearing triple-negative tumors (26).

In conclusion, both TNIK and p-TNIK were upregulated in PTC tissues, p-TNIK was significantly associated with extrathyroidal extension and both TNIK and p-TNIK may function as an oncogene to participate in the carcinogenesis and progression of PTC. Subsequent work will follow up the patients of the present study to explore the prognostic significance of TNIK and p-TNIK. Furthermore, *in vitro* and *in vivo* studies will be performed to elucidate the biological role and potential mechanism of TNIK, with the purpose of clarifying whether TNIK affects the biological behavior of PTC. In addition, whether TNIK functions as a critical oncogene in

PTC through Wnt/β-catenin or other biochemical pathways deserves further investigation and further experiments should be performed to determine whether TNIK inhibition may serve as a promising therapeutic approach for patients with PTC.

Acknowledgements

The authors would like to thank Professor Yueping Liu and Ms. Fei Lv (Pathology Department, The Fourth Hospital of Hebei Medical University, Shijiazhuang, China) for their technical assistance with the preparation of paraffin-embedded samples.

Funding

The present study was supported by the Beijing-Tianjin-Hebei Integration Project (grant no. J200004).

Availability of data and materials

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

Authors' contributions

JL: Execution of the majority of the experiments and manuscript writing. LL, YX and SL: Obtaining the tissue samples from the patients. ML, GH and ZW: Collection of clinicopathological data of patients. GW, YZ and JS: Statistical analysis of data. JW and YS: Extraction of RNA and execution of qPCR. RZ: Conception and design of the study, final review and supervision. GW and YZ confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The Ethics Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) approved this study (approval no. 2018MEC106).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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