

# Hypermethylation of the *RSK4* promoter associated with *BRAF V600E* promotes papillary thyroid carcinoma

YANAN YIN<sup>1</sup>, KUI CHE<sup>2</sup>, JIANXIA HU<sup>2</sup>, HUI HUA<sup>3</sup>, ANBING DONG<sup>3</sup>,  
JUERU WANG<sup>3</sup>, JIARUI YU<sup>1</sup>, QING ZHANG<sup>1</sup>, SHIHUA ZHAO<sup>1</sup>, YUHANG ZHAO<sup>1</sup>,  
PING WANG<sup>1</sup>, FEI WANG<sup>1</sup>, YANGANG WANG<sup>1</sup>, JINGWEI CHI<sup>2</sup> and WENHAI SUN<sup>3</sup>

<sup>1</sup>Department of Endocrinology and Metabolism, <sup>2</sup>Qingdao Key Laboratory of Thyroid Diseases;

<sup>3</sup>Department of Thyroid Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong 266000, P.R. China

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**Abstract.** Ribosomal S6 kinase 4 (*RSK4*) is a putative tumor suppressor gene which is inactivated by epigenetic events in a number human malignancies; however, its role in papillary thyroid carcinoma (PTC) remains largely unknown. The aim of this study was to explore the methylation status of the *RSK4* promoter in PTC, and to determine its potential role in thyroid carcinogenesis. Reverse transcription-quantitative PCR (RT-qPCR) and western blot analyses were performed to examine the *RSK4* mRNA and protein levels, respectively. Methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS) were used to analyze methylation status of the *RSK4* gene. Sanger sequencing was further carried out to detect the *BRAF V600E* mutation. Cell proliferation assay was finally performed to evaluate the role of hypermethylation in the growth of PTC cells. The association between *RSK4* methylation and the clinicopathological characteristics of patients with PTC was assessed. The methylation frequency of the *RSK4* promoter in PTC tissues was higher than that in paired paracancerous tissues. Coincidentally, the *RSK4* mRNA levels were also downregulated in PTC tissues when compared with the paracancerous counterparts. The hypermethylation of *RSK4* was associated with tumor size and lymph node metastasis. Furthermore, the *BRAF V600E* mutation may influence *RSK4* expression and methylation. Moreover, *RSK4* hypermethylation was observed in thyroid cancer cell lines, which was consistent with a lack of *RSK4* expression. Upon the

5-Aza-deoxycytidine treatment of thyroid cancer cells, *RSK4* expression was significantly upregulated, while cell proliferation was inhibited. On the whole, the findings of the present study demonstrate that the hypermethylation of the *RSK4* promoter may be one of the mechanisms responsible for the poor *RSK4* expression in PTC. Thus, these data suggest that *RSK4* may serve as a molecular target for the early diagnosis and treatment of PTC.

## Introduction

In recent decades, thyroid cancer has become a very common type of endocrine malignancy whose incidence has rapidly increased worldwide (1). As the most frequent histological type of thyroid cancer, the increased incidence of papillary thyroid carcinoma (PTC) has been pivotal to the increased numbers of patients affected by thyroid cancer (2). More sensitive reliable methods of diagnostic imaging and surveillance have also contributed to the higher detection of PTC (3). Despite the low mortality rate due to PTC, the disease recurrence is high (4). Therefore, exploring the potential molecular mechanisms responsible for carcinogenesis and the development of PTC is imperative.

Similar to other malignancies, thyroid cancer is a complex disease intertwined by multiple genetic and environmental factors (5). Recently, the reduced expression of ribosomal S6 kinase 4 (*RSK4*) has been implicated in the carcinogenesis of colon, kidney, breast, ovarian and endometrial cancers, suggesting a role as a putative tumor suppressor gene (6-10). *RSK4* belongs to the 90-kDa S6 ribosomal kinase family, and its coding gene is located on the X-chromosome (Xq21.1) (9). *RSK4* mediates signal transduction downstream of the mitogen-activated protein kinase (MAPK) cascades, serving as a serine/threonine kinase (11). Due to this kinase activity, *RSK4* also plays a significant role in the regulation of cellular survival, division and differentiation (9). It has been reported that *RSK4* overexpression is capable of inducing a senescence-like phenotype *in vitro*, while its downregulation can induce the escape from cellular senescence and, consequently, immortalization during the early stages of tumorigenesis (7). PTC is driven by multiple genetic elements, in which the *BRAF V600E* mutation is the most common

**Correspondence to:** Dr Wenhai Sun, Department of Thyroid Surgery, The Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Shinan, Qingdao, Shandong 266000, P.R. China  
E-mail: sunwenhaiqfy@163.com

Dr Jingwei Chi, Qingdao Key Laboratory of Thyroid Diseases, The Affiliated Hospital of Qingdao University, No. 16 Jiangsu Road, Shinan, Qingdao, Shandong 266000, P.R. China  
E-mail: cjw\_03@126.com

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oncogenic point mutation, occurring with a prevalence of 45% in PTC (12). *BRAF V600E* mutation aberrantly activates the MAPK pathway, and plays a crucial role in the carcinogenesis of thyroid cancer (13-15). Furthermore, *RSK4* is likely to act directly on or downstream of ERK, therefore inhibiting the MAPK signaling cascade (16).

Epigenetics can be described as a heritable modification of gene function that does not affect the genomic DNA sequence, although ultimately, it leads to a change in phenotype. In the mammalian genome, the methylation of gene promoters is one of the most common epigenetic events (17). As a common feature of tumorigenesis, hypermethylation can block the activity of certain genes which function as tumor suppressors, while demethylation can induce gene reactivation and expression (18). Therefore, the inactivation of gene transcription by promoter methylation may serve as a potential platform for the early diagnosis, risk and prognosis assessment, treatment and management of cancers (19).

The implication of *RSK4* methylation in human PTC has not been previously reported, at least to the best of our knowledge. Thus, the present study demonstrates that *RSK4* hypermethylation partly contributes to the downregulation of *RSK4* expression and is related to the tumor size and lymph node metastasis in PTC. Moreover, the present study investigated the association between *RSK4* and the *BRAF V600E* mutation in PTC. The present study indicates that *RSK4* potentially functions as a tumor suppressor and also participates in the carcinogenesis of PTC.

## Materials and methods

**Patients and tissue samples.** A total of 226 paired papillary thyroid carcinoma tissues and corresponding paracancerous counterparts were acquired between January, 2018 and February, 2019, after obtaining informed consents, from patients at the Affiliated Hospital of Qingdao University (Qingdao, China). For patients <18 years old, their written informed consents were provided by their parents. Tissue samples were collected, immediately frozen in liquid nitrogen and stored at -80°C until further analysis. All samples were confirmed by pathological assessment. In this study population, 169 patients were female and 57 were male. The median age at diagnosis was 45 years (range, 17-76 years). Patients with primary PTC were included and those who had received chemotherapy or radiotherapy prior to surgery were excluded. The study design and implementation were approved by the Ethics Committee of the Affiliated Hospital of Qingdao University.

**Cells and cell culture.** Immortalized thyroid cancer cell lines (TPC-1 and BHT101) and a normal thyroid epithelial cell line (Nthy-ori3-1) were obtained from ExPASy Bioinformatics Resource Portal and Type Culture Collection of the Chinese Academy of Sciences, respectively. TPC-1 and BHT101 cells were cultured in DMEM supplemented with 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc.). Nthy-ori3-1 cells were cultured in F12K medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. The cells were maintained in incubator at 37°C and 5% CO<sub>2</sub>. When required, the TPC-1, BHT101 and Nthy-ori3-1 cells were treated with

10  $\mu$ M 5-Aza-deoxycytidine (5-Aza; Sigma-Aldrich; Merck KGaA) for 5 consecutive days.

**Reverse transcription-quantitative RCR (RT-qPCR).** Total RNA was extracted using TRIzol reagent (T9424, Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Total RNA (3  $\mu$ g) was reverse transcribed at 37°C for 15 min and 85°C for 5 sec using the PrimeScript™ RT reagent kit with gDNA Eraser (Code no. RR047, Takara Bio, Inc.). Subsequently, single-stranded cDNAs were subjected to qPCR using TB Green® Premix Ex Taq™ (Code no. RR420, Takara Bio, Inc.) on a LightCycler® 96 Real-Time PCR System (Cobas z480, Roche). The amplification conditions were as follows: 95°C for 30 sec, followed by 30 cycles of 95°C for 5 sec and 60°C for 30 sec. The following gene-specific qPCR primers were used in this study: *RSK4* forward, 5'-GAACAGACATTTCCAGCTAAAAAGG-3' and reverse, 5'-TGCCTGAAGATCCAGCACTAA-3'; *BRAF* forward, 5'-TGGGGAACGGAAGTGAATTTTC-3' and reverse, 5'-TTTTGTGGTGACTTGGGGTTG-3'. GAPDH was used as a reference gene (normalizer) and amplified using the following primers: Forward, 5'-TGCACCACCAACTGCTTAGC-3' and reverse, 5'-GGCATGGACTGTGGTCATGAG-3'. The relative mRNA expression of *RSK4* was analyzed using the 2<sup>- $\Delta\Delta C_q$</sup>  method (20).

**Western blot analysis.** Cell samples were washed twice with PBS and then lysed in Laemmli buffer (2% SDS, 10% glycerol, 0.125M Tris-Cl, pH 6.8). The BCA method was used for protein quantification. The concentrations of separating gel and stacking gel were 10 and 5%. Equal amounts of total protein (20  $\mu$ g) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk and incubated with respective primary antibodies overnight at 4°C. The membranes were washed 3 times with TBST buffer (containing 0.1% Tween-20) and then incubated with corresponding HRP-conjugated secondary antibody for 1 h at room temperature. The membranes were washed again, 3 times with TBST, prior to protein detection. Specific protein bands were developed by chemiluminescence using a commercial ECL kit (Millipore). The following antibodies were used for western blot analysis: Mouse anti-*RSK4* monoclonal antibody (1:500, 27330, Novus Biologicals); rabbit anti-GAPDH polyclonal antibody (1:2,000, E-AB-20059, Elabscience Biotechnology); the secondary antibodies were as follows: Goat anti-rabbit/goat anti-mouse IgG polyclonal antibody (1:10,000, 111-035-003/115-005-003, Jackson ImmunoResearch Laboratories).

**DNA extraction and sodium bisulfite modification.** Total genomic DNA was extracted from the tissues and cells using a Genomic DNA extraction kit (Aidlab). All procedures followed according to manufacturer's protocol. Genomic DNA samples (3  $\mu$ g each) was bisulfite-modified and then purified according to the manufacturer's protocol. (Qiagen).

**Methylation-specific PCR (MSP).** In total, 134 pairs of specimens of PTC patients were included. Specific primers of *RSK4* for the unmethylated (5'-TTTTTATTGATGTTTGGGTGAT-3' and 5'-AACAACAACCACTCAATAATAAC-3') and methylated reactions (5'-TTATTGACGTTTGGGTGAC-3'

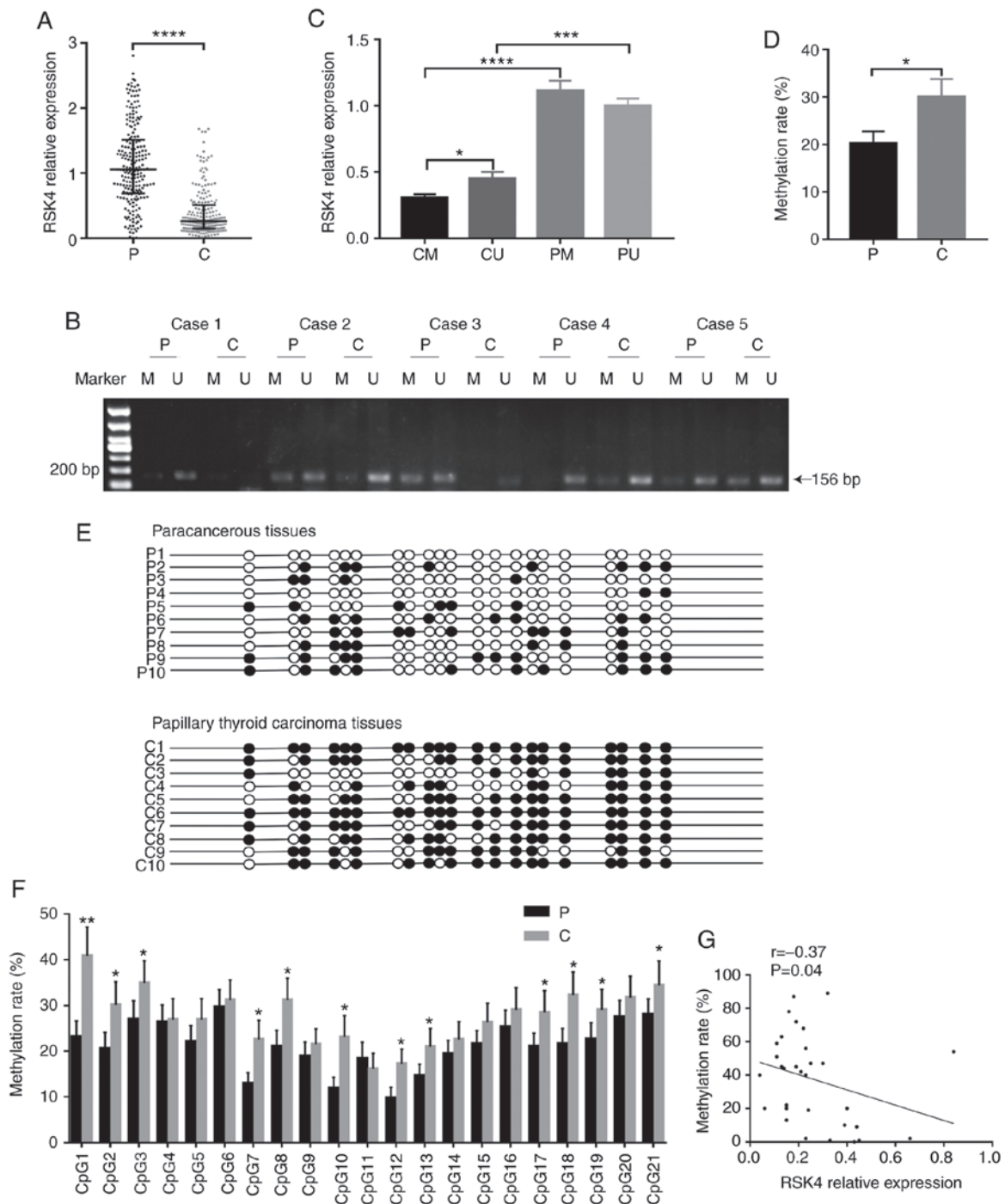


Figure 1. *RSK4* is downregulated and its promoter region is hypermethylated in PTC tissues. (A) *RSK4* expression in PTC tissues and paired paracancerous tissues, analyzed by RT-qPCR (n=226). (B) Representative agarose gel electrophoretogram showing different types of MSP products (P, paracancerous tissues; C, PTC tissues; U, unmethylated; M, methylated). (C) *RSK4* expression in different groups, analyzed by RT-qPCR (CU, unmethylated PTC tissues; CM, methylated PTC tissues; PU, unmethylated paracancerous tissues; PM, methylated paracancerous tissues). (D) Methylation rate in PTC tissues and paired paracancerous tissues, analyzed by BGS. (E) BGS analysis of methylation in *RSK4* promoter. A total of 21CpG sites of 10 patients were randomly selected from 31 patients analyzed by BGS. The black and white circles indicate the methylated and unmethylated CpG sites, respectively. (F) Methylation ratio of each CpG sites, identified in the sequenced *RSK4* promoter region from PTC and paired paracancerous tissues. (G) Analysis of correlation between *RSK4* mRNA expression and the methylation rates in PTC tissues. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ , compared to paracancerous tissues or as indicated. *RSK4*, ribosomal S6 kinase 4; PTC, papillary thyroid carcinoma; MSP, methylation-specific PCR; BGS, bisulfite genomic sequencing.

and 5'-GACAACCGCTCGATAATAAC-3') were utilized. The modified DNA was amplified by PCR using the following conditions: 98°C for 5 min, followed by 35 cycles of 98°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec, and a final 5-min incubation at 72°C. PCR products were isolated by agarose gel electrophoresis.

**Bisulfite sequencing (BGS).** In total, 31 pairs of specimens of PTC patients were included. Modified DNA samples were subjected to PCR amplification to further analyze the methylation status of the *RSK4* promoter. The primers used for bisulfite genomic sequencing were as follows: Forward, 5'-TTTTAA GTTGGGGAATTTTATTGA-3' and reverse, 5'-AAAACA

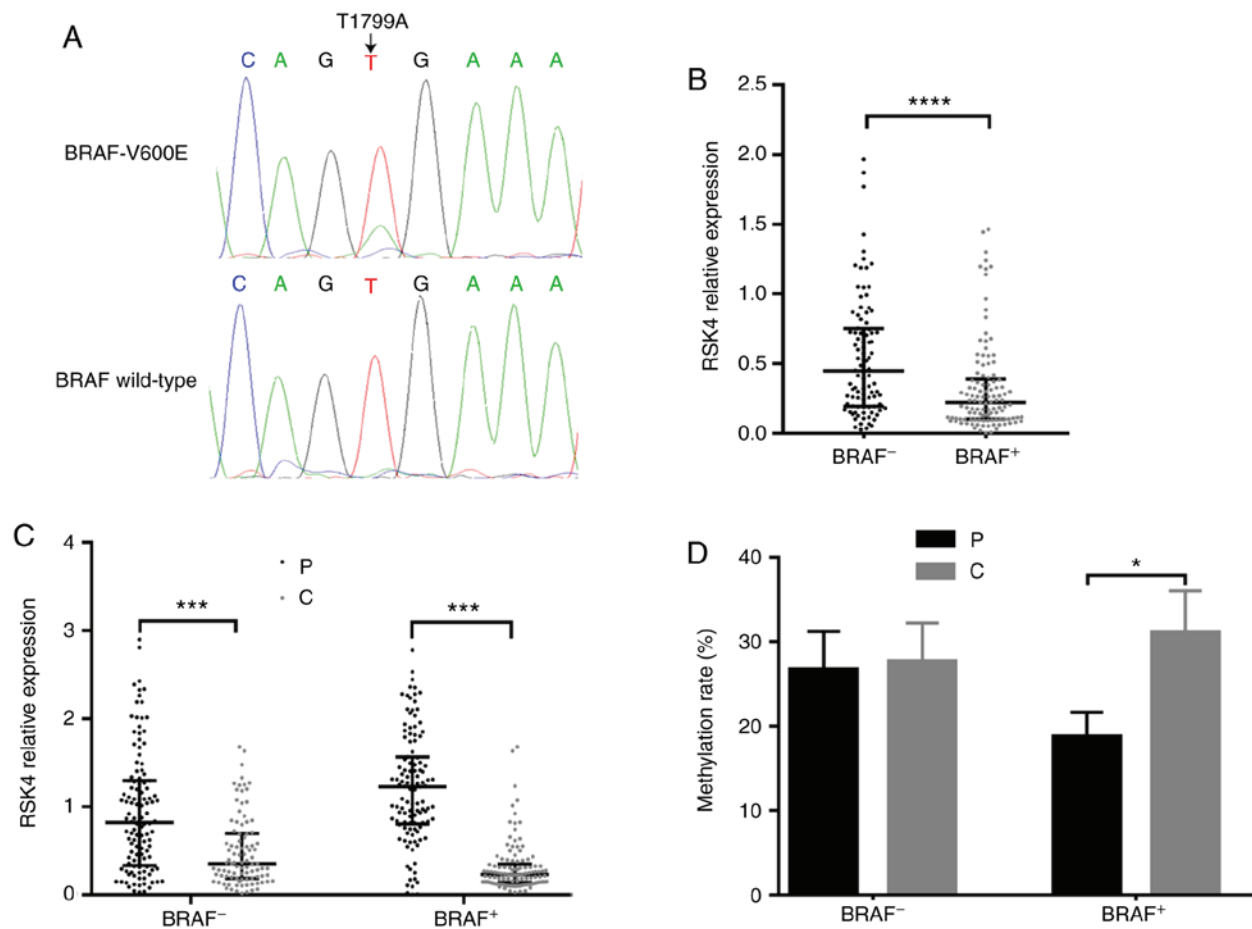


Figure 2. Association between *BRAF* V600E mutation with *RSK4* expression and methylation in PTC tissues. (A) Sequence chromatograms of PTC DNA with non-mutated *BRAF* and *BRAF* V600E mutation. (B) *RSK4* mRNA levels (PTC relative to paracancerous tissues) in patients with non-mutated *BRAF* (*BRAF*<sup>-</sup>) and *BRAF* V600E mutation (*BRAF*<sup>+</sup>). (C) *RSK4* expression in PTC and paracancerous tissues, grouped by *BRAF*. (D) BGS methylation rate in PTC and paracancerous tissues, grouped by *BRAF*. \**P*<0.05; \*\*\**P*<0.001; \*\*\*\**P*<0.0001. *RSK4*, ribosomal S6 kinase 4; PTC, papillary thyroid carcinoma; BGS, bisulfite genomic sequencing.

ACTCTATACTACCTCTCCAAAAAC-3' (21). DNA samples were amplified by PCR using the following conditions: 98°C for 5min, followed by 35 cycles of 98°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec, and a final 5-min incubation at 72°C. PCR products were ligated into the pMD18-T Vector (Takara Bio, Inc.), followed by transformation into competent the *Escherichia coli* strain, DH5a (Takara Bio, Inc.). At least 6 clones from each ligation reaction were randomly selected. Bacterial solutions were then sequenced by the Beijing Genomics Institute (China). The percentage of methylated DNA for each sample was computed according to the number of methylated CpG dinucleotides/(6x21)x100%.

***BRAF* V600E mutation analysis.** The hot spot region of *BRAF* V600E mutation was amplified using Premix Taq™ (Takara, Code No. R004). The following PCR primers were used: Forward, 5'-GCTTGCTCTGATAGGAAAATGAG-3' and reverse, 5'-GTAAGTCTCAGCAGCATCTCAGG-3'. Genomic DNA was amplified by PCR using following conditions: 95°C for 3 min, followed by 38 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 45 sec, and a final 10-min incubation at 72°C. Respective PCR products were sequenced by the Beijing Genomics Institute to confirm the *BRAF* V600E mutation.

**Cell transfection.** siRNAs targeting *BRAF* (5'-GCAUCA AUG GAUACCGUUA-3') and control siRNAs (5'-UUCUCCGAA CGUGUCACGU-3') were synthesized by GenPharma. To knockdown *BRAF* V600E, siRNAs were transfected into the BHT101 cells using HiPerFect Transfection Reagent (Qiagen), according to the manufacturer's protocol. Until transfection, the cells were incubated at 37°C and 5% CO<sub>2</sub>, and the transfection complexes which had been incubated at room temperature for 10 min were then added to the cells. After 24 h, the cells were harvested and total RNA was extracted.

**Cell proliferation assay.** The proliferation of the TPC-1 and BHT101 cells was measured using the cell counting kit-8 (MedChemExpress). For this, cells were seeded into 96-well culture plates (2x10<sup>3</sup> cells/well) and cultured accordingly for 24 h. CCK-8 reagent was added (10 μl/well) at the 0, 24, 48, 72, 96 h followed by incubation for an additional 2 h at 37°C. The absorbance was measured at 450 nm using an enzyme immunoassay analyzer (Gen5 software, BioTek Eliasa).

**Statistical analysis.** Statistical analysis was performed in silico using SPSS (version 23.0. IBM) and Prism 7 (GraphPad Software Inc.). Data with normal distribution are presented as the means ± SEM. Otherwise, data are expressed as the median



Table I. Association between the *RSK4* methylation rate and clinicopathological features of patients with PTC.

Variable	No. of patients	Methylation rate in PTC tissues	P-value
Sex			
Male	16	29.86±4.784	0.1324
Female	15	18.47±5.503	
Age (years)			
<55	25	28.63±4.134	0.7537
≥55	6	31.75±10.490	
Tumor size (cm)			
≤1	13	26.68±5.405	0.5785
>1	18	31.08±5.392	
Lymphatic metastasis			
Present	17	37.93±6.038	0.0369
Absent	14	22.08±4.304	
Invasion			
Present	8	24.01±8.946	0.4293
Absent	23	31.06±4.176	
TNM stage			
I	20	30.42±3.795	0.4296
II	11	21.23±16.31	
Hashimoto thyroiditis			
Present	9	31.48±8.938	0.8512
Absent	22	29.00±4.163	

with the first and third interquartile range (Q1, Q3). Paired samples from the same patients were compared using the paired Student's t-test. An unpaired t-test or two-tailed Mann Whitney U test was used to analyze the difference between 2 groups. Multiple groups (≥3) were compared using one-way analysis of variance ANOVA and the Bonferroni multiple comparisons test. Spearman's correlation analysis was used to analyze the correlation between *RSK4* mRNA expression and the methylation rates in PTC tissues. The difference of rate was analyzed using the Chi-square test.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

**Reduced *RSK4* expression and hypermethylation of its promoter region in PTC.** To verify whether the inactivation of *RSK4* may contribute to thyroid carcinogenesis, *RSK4* expression in PTC tissues and paracancerous counterparts was analyzed by RT-qPCR. The *RSK4* mRNA levels were notably reduced in the PTC tissues when compared with their counterparts ( $n=226$ ,  $P<0.0001$ ) (Fig. 1A).

In order to assess the possible mechanism of the down-regulation of *RSK4* levels in PTC, the methylation status of its gene was examined. A 240-bp region, including 21 CpG sites located in the *RSK4* promoter, was analyzed. MSP and BGS analyses were performed to investigate whether a promoter-specific CpG methylation was associated with the decreased *RSK4* expression. MSP analysis revealed that the methylation rates of *RSK4* in the PTC tissues (93/134, 69.40%)

were higher than those in the paracancerous tissues (75/134, 55.97%,  $P<0.05$ ). *RSK4* expression in methylated PTC tissues ( $n=93$ ) was reduced compared with that in unmethylated PTC tissues ( $n=41$ ,  $P<0.05$ ) (Fig. 1C), with no significant difference in expression in the paracancerous tissues ( $P>0.05$ ). *RSK4* expression in methylated PTC tissues was lower than that in methylated paracancerous tissues ( $n=74$ ,  $P<0.0001$ ). Moreover, *RSK4* expression in unmethylated PTC tissues was also lower than that in unmethylated paracancerous tissues ( $n=60$ ,  $P<0.001$ ) (Fig. 1C).

BGS analysis also provided evidence that the methylation rates were higher in PTC tissues ( $n=31$ ,  $P<0.05$ ) (Fig. 1D and E). In the majority of CpG sites, the methylation rates of the *RSK4* promoter were higher in PTC than in paracancerous tissues (Fig. 1F). In addition, it was found that *RSK4* mRNA expression negatively correlated with the methylation rates in PTC tissues ( $r=-0.37$ ,  $P<0.05$ ) (Fig. 1G). Taken together, these results indicate that the methylation rates of the *RSK4* promoter are increased in PTC, leading to the downregulation of *RSK4* expression.

**Association between *BRAF* V600E mutation, and the expression and methylation of *RSK4* in PTC.** Previous studies have revealed that *RSK4* is involved in MAPK signaling transduction and, therefore, it can regulate ERK activation. Thus, the present study investigated whether there was any association between the *BRAF* V600E mutation with *RSK4* expression and methylation in PTC. The chromatograms of DNA sequencing are presented in Fig. 2A, denoting *BRAF* T1799A and

Table II. Association between the *RSK4* methylation status and clinicopathological features of patients with PTC with or without *BRAF* V600E mutation.

Variable	BRAF <sup>+</sup>				BRAF <sup>-</sup>			
	No. of patients	Methylated	Unmethylated	P-value	No. of patients	Methylated	Unmethylated	P-value
Sex								
Male	23	15	8	0.2906	12	5	7	0.0586
Female	48	37	11		51	36	15	
Age (years)								
<55	60	44	16	0.9667	55	36	19	0.8699
≥55	11	8	3		8	5	3	
Tumor size (cm)								
≤1	30	18	12	0.0311	36	22	14	0.4455
>1	41	34	7		27	19	8	
Lymphatic metastasis								
Present	44	36	8	0.0371	31	22	9	0.3346
Absent	27	16	11		32	19	13	
Invasion								
Present	16	10	6	0.2703	9	4	5	0.1607
Absent	55	42	13		54	37	17	
TNM stage								
I	58	42	16	0.7399	55	36	19	0.8699
II	13	10	3		8	5	3	
Hashimoto thyroiditis								
Present	10	6	4	0.3076	19	11	8	0.4318
Absent	61	46	15		44	30	14	

wild-type *BRAF*. It was found that the *RSK4* mRNA levels (PTC relative to the paracancerous tissues) in PTC patients with *BRAF* V600E mutation (n=124) were significantly lower than those with wild-type *BRAF* (n=102,  $P<0.0001$ , Fig. 2B). Independently of the *BRAF* mutation status, the *RSK4* mRNA levels in PTC tissues were lower than those in paracancerous tissues (Fig. 2C). However, only in patients with *BRAF* V600E mutation, the methylated rates in the PTC tissues were higher than those in paracancerous tissues, as detected by MSP (73.23 vs. 50.7%,  $P<0.001$ ) and BGS ( $P<0.05$ ) (Fig. 2D). Taken together, these findings indicate that *BRAF* V600E mutation may be one of reasons for the downregulation of *RSK4* mRNA levels due to the increasing methylation rates in PTC.

*Association between RSK4 methylation levels and the clinicopathological characteristics of patients with PTC.* To further explore the role of *RSK4* hypermethylation in PTC, 134 (MSP) and 31 (BGS) PTC patients with available information were analyzed. BGS analysis revealed that the methylation rates were higher in tumors with lymph node metastasis (Table I). MSP analysis revealed that the methylation rates in PTC tumors >1.0 cm in diameter (34/41, 82.93%) were higher than those in PTC tumors ≤1.0 cm in diameter (18/30, 60%) in patients with *BRAF* V600E mutation (Table II). In addition, the methylation rates were higher in tumors with lymph node metastasis (36/44, 81.82%)

compared with those without metastasis (16/27, 59.26%) in patients with *BRAF* V600E mutation (Table II). PTC patients with Hashimoto thyroiditis were also included to examine whether there was an association between the methylation status of *RSK4* and Hashimoto thyroiditis in PTC; it was found that the methylation rate of *RSK4* was not related to Hashimoto thyroiditis (Tables I and II). These findings are in accordance with a tumor suppressor role for *RSK4* in inhibiting tumor growth and metastasis in PTC.

*RSK4 expression associated with BRAF V600E is down-regulated and hypermethylated in thyroid cancer cells.* *RSK4* levels and the methylation status of its promoter were examined *in vitro*, using a normal (Nthy-ori3-1) and thyroid cancer cell lines (TPC-1 and BHT101). The results revealed higher methylation rates of the *RSK4* promoter in TPC-1 and BHT101 when compared with Nthy-ori3-1 cells (Fig. 3C). By contrast, both the *RSK4* mRNA and protein levels were lower in the TPC-1 and BHT101 cells when compared with the Nthy-ori3-1 cells (Fig. 3A and B). As shown in Fig. 3D and E, *BRAF* expression was significantly downregulated and *RSK4* expression was significantly upregulated following the knock-down of *BRAF* V600E in the BHT101 cells. These findings suggest that *RSK4* expression is negatively associated with the methylation status of its promoter and is also influenced by *BRAF* V600E in thyroid cancer cells. These *in vitro* data

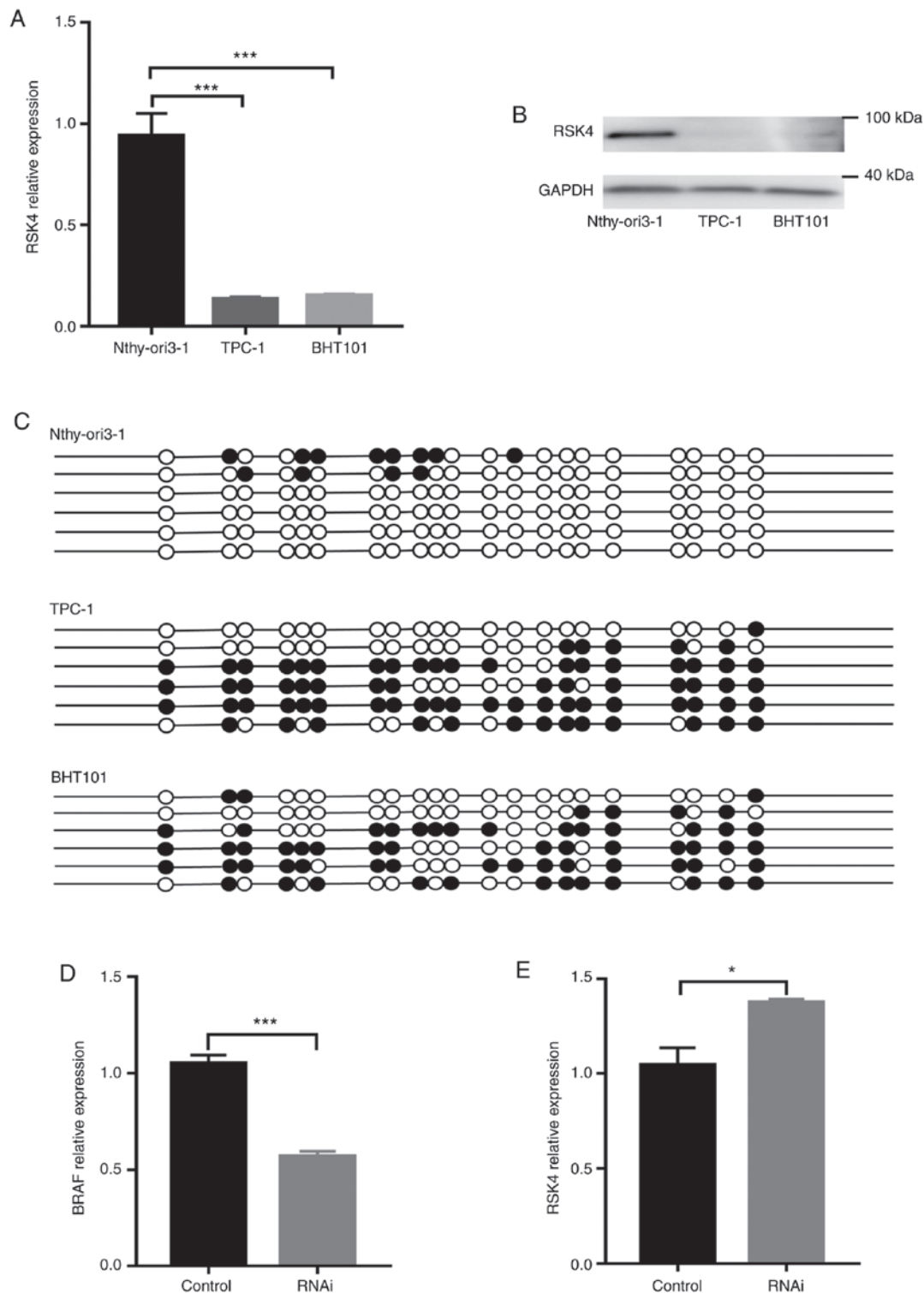


Figure 3. RSK4 expression and methylation status in Nthy-ori3-1, TPC-1 and BHT101 cell lines. (A) *RSK4* mRNA levels in Nthy-ori3-1, TPC-1 and BHT101, detected by RT-qPCR. (B) RSK4 protein levels in Nthy-ori3-1, TPC-1 and BHT101 cells, detected by western blot analysis. GAPDH was used as an internal control. (C) BGS analysis of methylation in *RSK4* promoter from 21 CpG sites of 6 clones from Nthy-ori3-1, TPC-1 and BHT101 cells. The black and white circles indicate the methylated and unmethylated sites. (D and E) BRAF and RSK4 expression in BHT101 cells, detected by RT-qPCR following transfection with siRNA targeting BRAF. \*P<0.05; \*\*\*P<0.001. RSK4, ribosomal S6 kinase 4; BGS, bisulfite genomic sequencing.

are consistent with the results obtained with the human tissue specimens mentioned above.

*RSK4* expression is enhanced by 5-Aza and DNA demethylation inhibits the proliferation of thyroid cancer cells. To further investigate the role of promoter hypermethylation in

the regulation of RSK4 expression, the TPC-1, BHT101 and Nthy-ori3-1 cells were treated with the demethylating agent, 5-Aza. The results revealed that the *RSK4* mRNA and protein levels were restored in the TPC-1 and BHT101 cells, but not in the Nthy-ori3-1 cells (Fig. 4A and B). Furthermore, the proliferative ability of the TPC-1 and BHT101 cells was significantly

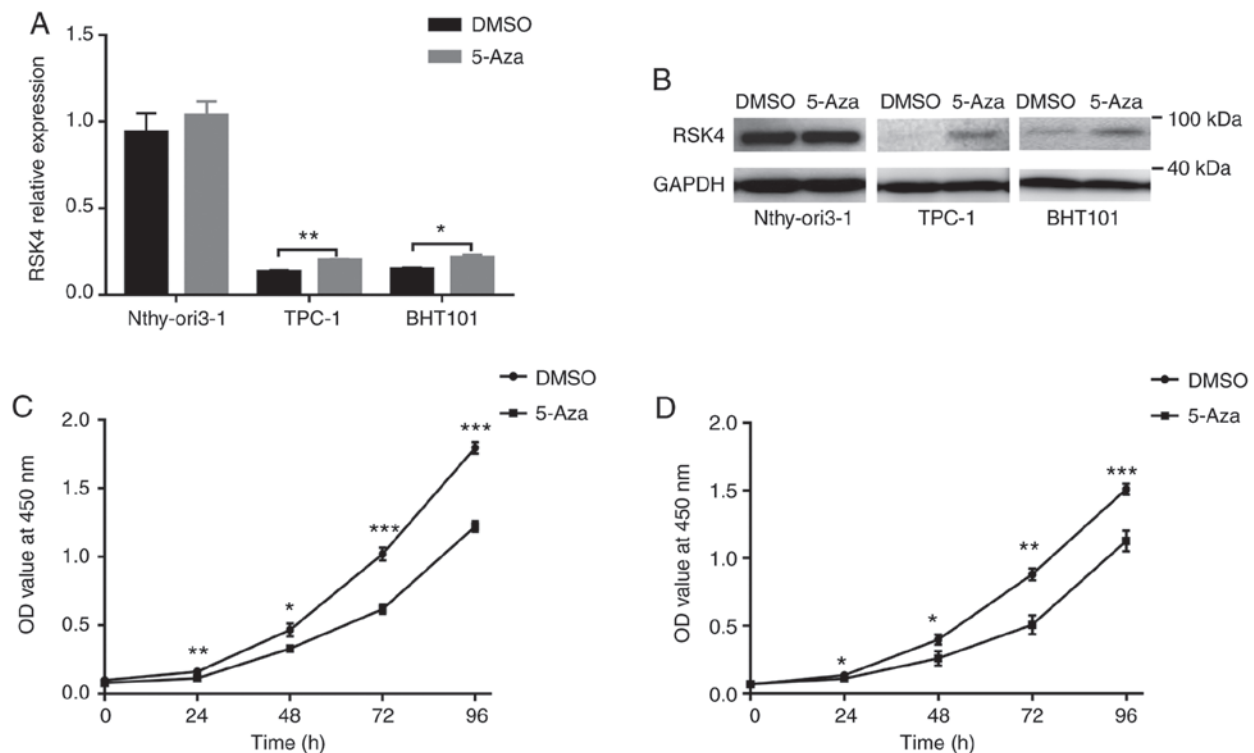


Figure 4. RSK4 expression and cell proliferation upon treatment with the demethylating agent, 5-Aza. (A and B) *RSK4* mRNA and protein levels, analyzed by RT-qPCR and western blot analysis in Nthy-ori3-1, TPC-1 and BHT101 cells treated (or not) with 10  $\mu$ M 5-Aza. DMSO treatment was performed as a negative control. Proliferation of (C) TPC-1 and (D) BHT101 cells, determined by CCK-8 assay following treatment with 10  $\mu$ M 5-Aza (using DMSO as a control). \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001. RSK4, ribosomal S6 kinase 4; 5-Aza, 5-Aza-deoxycytidine.

suppressed by treatment with 10  $\mu$ M 5-Aza (Fig. 4C and D). These data suggest that the hypermethylation of the *RSK4* promoter can reduce gene expression and, importantly, DNA demethylation can lead to the repression of thyroid cancerous cell proliferation.

## Discussion

Papillary thyroid cancer (PTC) accounts for 80-90% of all thyroid cancers and is the most common endocrine malignancy, with a rapidly increasing incidence worldwide (22). Although thyroid cancer has been frequently associated with a relatively indolent progress and a good prognosis, some patients become incurable with notable morbidity and mortality (3,23). In spite of the overall 10-year survival rate (approximately 90%), approximately 10-20% of patients with PTC with stage I or II disease suffer from disease recurrence (24). The molecular pathogenesis of thyroid cancer involves genetic alterations of multiple oncogenes, such as *BRAF*, *TERT* and *RAS*, and tumor suppressor genes, such as *PTEN* and *RASAL1*. Their potential association with clinicopathologic aggressiveness and the poor prognosis of thyroid cancer has been partially demonstrated (12,14,25). Advances in the molecular pathogenesis of thyroid cancer have exhibited great prospect toward more effective diagnosis and treatment strategies for this disease (12). Moreover, the understanding of these genetic alterations may further lead to a reduction in unnecessary treatments for indolent PTC, and may also improve the prognosis of patients affected by clinically aggressive cancers (3).

*RSK4* is a functional ribosome protein kinase that belongs to the p90 RSK family. Recent studies have demonstrated its apparent significance in tumorigenesis, since a low or no *RSK4* expression has been associated with malignant tumors of the colon, kidney, ovary, pancreas, hematological system and endometrium (8,9,26-28). Specifically, it has been demonstrated that *RSK4* may drive cellular senescence by modulating Rb and p21, since the upregulation of *RSK4* expression can increase p21 levels (6,7,29). These studies have indicated that the downregulation of *RSK4* may be an important element in promoting cell transformation and, therefore, may support the notion of the role of *RSK4* in regulating senescence as tumor suppressor protein (6-8,16). Similarly, the present study found that *RSK4* expression was significantly decreased in the majority of PTC tissues and in a PTC-derived cell line.

Promoter hypermethylation has been recognized as standard mechanism that leads to the transcriptional inactivation of tumor suppressor genes (TSGs) in a variety of cancer types (30). Some studies have indicated that the hypermethylation of the *RSK4* promoter can lead to a decrease in its own expression in several malignancies, including breast, endometrial, oral and ovarian cancers. Accordingly, the decreased *RSK4* expression has been restored by DNA methyltransferase inhibitors in several cancer cell lines (21,27,30,31). However, no studies to date have reported the methylation status of *RSK4* in PTC and paracancerous tissues, at least to the best of our knowledge. Hence, the authors conducted MSP and BGS to finally examine the methylation status of *RSK4*. Both approaches were able to demonstrate that the methylation



rates of *RSK4* promoter were consistently higher in PTC than in paracancerous tissues. This increased methylation status negatively correlated with *RSK4* expression in PTC tissues. Upon methylation, the *RSK4* mRNA levels in PTC were lower than those in paracancerous tissues. Under non-methylating conditions, the *RSK4* mRNA levels were less prominently decreased in PTC when compared with those in paracancerous tissues. As stated, it appears that *RSK4* methylation may coexist in both PTC and paracancerous tissues, where promoter methylation may be one of the various mechanisms that can lead to a reduction in *RSK4* expression. According to the clinicopathological data, this study also demonstrated the association between *RSK4* promoter methylation with lymph node metastasis and tumor size, providing evidence that *RSK4* methylation may play a role in the development and prognosis of PTC. Indeed, previous studies have indicated that *RSK4* can suppress tumor growth, invasiveness and metastatic ability of other cancer cell lines (7,8).

Strikingly, the aberrant *RSK4* expression in PTC cells could be reversed by broad-spectrum demethylated drugs, and DNA demethylation inhibited the proliferation of thyroid cancer cells. In other words, the tumor suppressor effect of *RSK4*, to a certain extent, could be restored by demethylation, suggesting that targeted demethylation drugs may be an attractive option for tumor therapy in the near future.

An extensive number of genetic alterations have been involved in the tumorigenesis of various thyroid cancers. The T1799A transverse point mutation of *BRAF* is a prototypical example, which leads to the expression of a *BRAF V600E* mutant protein and contributes to constitutive activation of this serine/threonine kinase. *BRAF V600E* mutation can activate the MAPK signaling pathway, which is a key feature of PTC (12,32). A previous comprehensive and multicenter study demonstrated a strong association between *BRAF V600E* with the poor clinicopathological outcomes of PTC, including aggressive pathological characteristics, disease recurrence, loss of radioiodine avidity and unfavorable prognosis (23,33-35). Previous studies have demonstrated that *RSK4* can negatively modulate the MAPK pathway at the ERK or the downstream levels, depending on its kinase activity (10,26). The present study analyzed the status of *BRAF* mutations in 226 PTC tissues and, as a result, it was found that *BRAF V600E* mutation rates were considerably high. Furthermore, this study indicates that *BRAF V600E* mutation may influence *RSK4* expression, leading to a more significant difference on mRNA levels between PTC tissues and paracancerous tissues. Moreover, it was found that this mutation can influence the methylation status of *RSK4*, thereby influencing the clinicopathological outcomes of patients with PTC, which reiterates the putative role of *RSK4* as a cancer suppressor gene in thyroid tumors.

To conclude, the present study demonstrates that the hypermethylation of CpG islands is one prominent mechanism that can lead the downregulation of *RSK4* expression. The hypermethylation of the *RSK4* promoter is associated with tumor proliferation and lymph node metastasis. *BRAF V600E* mutation also has an effect on *RSK4* mRNA levels and gene methylation status, supporting the distinctive role of *RSK4* in the development, progression and prognosis of PTC.

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## Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Authors' contributions

YY, YW, JC and WS designed the study and wrote the manuscript. YY, JC, KC and WS performed the majority of the experiments. YW, JH, HH, AD, JW, QZ, JY, SZ, YZ, PW and FW participated in the experimentation. WS provided the patients and participated in the discussions. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Affiliated Hospital of Qingdao University (QDFY WZ 2018-1-14-02) and was conducted in accordance with the Helsinki Declaration of 1964 and its later amendments or comparable ethical standards. All patients provided written informed consent. For patients <18 years old, their written informed consents were provided by their parents.

## Patient consent for publication

Not applicable.

## Competing interests

All authors declare that they have no competing interests.

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