

Original Article

***RUNX3* site-specific hypermethylation predicts papillary thyroid cancer recurrence**

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Abstract: Papillary thyroid cancer (PTC) is the most common epithelial thyroid tumor, accounting for more than 80% of all thyroid cancers. Although PTC shows an indolent character and excellent prognosis, patients with aggressive characteristics are more likely to have a disease recurrence and die in the end. The aim of this study was to analyze *BRAF*^{V600E} mutation and methylation levels of CpG sites in the promoters of *CDH1*, *DAPK*, *RARβ* and *RUNX3* genes in a cohort of PTCs, and investigate their association with tumor recurrence. In this study, we used pyrosequencing method to individually quantified methylation levels at multiple CpG sites within each gene promoter, and detect *BRAF*^{V600E} mutation in 120 PTCs and 23 goiter tissues as normal control. Moreover, appropriate cut-off values for each CpG site were set up to predict disease recurrence. Our data showed that overall average methylation levels of *CDH1* and *RUNX3* genes were significantly higher in PTCs than that in control subjects. Conversely, overall average methylation levels of *DAPK* promoter were significantly lower in PTCs than that in control subjects. Moreover, *BRAF*^{V600E} mutation and overall average methylation levels of all these genes were not significant difference between recurrent and non-recurrent cases. However, we found that hypermethylation of *RUNX3* at CpG sites -1397, -1406, -1415 and -1417 significantly increased the risk of of disease recurrence by using appropriate site-specific cut-off values. Collectively, our findings suggest *RUNX3* site-specific hypermethylation may offer value in predicting or monitoring postoperative recurrence of PTC patients.

Keywords: Papillary thyroid cancer (PTC), tumor recurrence, *RUNX3*, promoter hypermethylation, pyrosequencing

Introduction

Thyroid cancer is the most common endocrine malignancy, the incidence of which has increased continuously in recent years. The most common histological type is particularly papillary thyroid cancer (PTC), which accounts for more than 80% of all thyroid cancers [1, 2]. Although the majority of PTCs can be efficiently cured by surgical and radioiodinated therapy and have an excellent prognosis, recurrent disease develops in about 20% to 30% of patients with lymph node metastasis, and ~7% of patients die from progressive disease within 10 years of diagnosis [3]. Therefore, there is pressing need to develop an effective strategy for predicting or monitoring tumor recurrence before or after initial surgery. It would be clinically important for improving survival and quality of life in patients with PTC.

It is well known that *BRAF*^{V600E} mutation is the most prominent oncogenic event in PTC, which results in constitutively activated BRAF kinase [4]. It plays a critical role in PTC tumorigenesis through driving Ras/Raf/MEK/ERK (MAPK) signaling [4, 5]. However, the association of *BRAF*^{V600E} mutation with PTC recurrence remains controversial [6, 7]. In addition, DNA methylation, one of epigenetic changes, is also the most extensively studied in diverse human cancers and can be used as potential biomarkers for cancer diagnosis, including PTC [8]. Generally, DNA methylation as an important hallmark of cancer cells is one of the major mechanisms to inactivate tumor suppressor genes and seriously affect their function, contributing to carcinogenesis [9, 10]. It has been demonstrated that promoter hypermethylation of certain genes is associated with disease recurrence in diverse cancers, including blad-

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der cancer, non-small cell lung cancer, prostate cancer and hepatocellular carcinoma [11-14].

In this study, we investigated methylation levels of *CDH1*, *DAPK*, *RARβ* and *RUNX3* genes and *BRAF*^{V600E} mutation in a cohort of PTC samples by using pyrosequencing method, and attempted to discover CpG site-specific hypermethylation for accurately predicting disease recurrence and determine the association of *BRAF*^{V600E} mutation with PTC recurrence.

Material and methods

Patients and tissue samples

With the approval of our institutional review board and human ethics committee, a total of 120 paraffin-embedded PTCs and 23 goiter tissues used as control subjects were randomly obtained at the First Affiliated Hospital of Xi'an Jiaotong University School of Medicine. None of these patients received chemotherapy and radiotherapy before the surgery. Informed consent was obtained from each patient before the surgery. All paraffin-embedded tissues were examined and agreed upon by a senior pathologist at Department of Pathology of the Hospital. Clinicopathologic data, including gender, age, lymph node metastasis, tumor stage and recurrence, was collected retrospectively, and was summarized in [Supplementary Table 1](#).

DNA isolation

Genomic DNA was extracted from isolated tissues as previously described [19]. Briefly, after a treatment for 12 h at room temperature with xylene to remove paraffin, the tissues were digested with 1% sodium dodecyl sulfate (SDS) and 0.5 mg/ml proteinase K at 48°C for 48 h, with addition of several spiking aliquots of concentrated proteinase K to facilitate digestion. Genomic DNA was then isolated from the digested tissues using standard phenol/chloroform protocol, and was dissolved in distilled water and stored at -80°C until use.

Sodium bisulfite treatment

DNA from thyroid tissues and cell lines was subjected to bisulfite treatment as described previously [15]. Briefly, a final volume of 20 μl of H₂O containing ~2 μg genomic DNA, 10 μg salmon sperm DNA, and 0.3 M NaOH was incu-

bated at 50°C for 20 min to denature the DNA, followed by incubation in a 500 μl reaction mixture freshly prepared containing 3 M sodium bisulfite (Sigma, Saint Louis, MO) and 10 mM hydroquinone (Sigma, Saint Louis, MO) at 70°C for 3 h. The DNA was then purified by a Wizard DNA Clean-Up System (Promega Corp., Madison, WI) according to the instructions of the manufacturer, followed by ethanol precipitation, and resuspension in 50 μl of deionized H₂O after vacuum drying for DNA methylation analysis. Bisulfite-treated DNA samples were stored at -80°C until use.

DNA methylation analysis using pyrosequencing

We used bisulfite pyrosequencing assay to quantitatively analyze methylation levels of 4 genes, including *CDH1*, *DAPK*, *RARβ* and *RUNX3*. The amplifying and sequencing primers were designed using PyroMark Assay design software ver. 2.0 (Qiagen, Valencia, CA), and the sequences were presented in [Supplementary Table 2](#). The PCR was conducted in a volume of 30 μl with ~30 ng bisulfite-converted DNA, 1×PCR buffer, 3 mM MgCl₂, 300 μM each of deoxynucleotide triphosphate mixture (dATP, dCTP, dGTP and dTTP), 300 nM forward and reverse primers, and 1.0 U of platinum Taq DNA polymerase (Invitrogen Technologies, Inc., CA). The amplification was performed in a Thermal cycler (Bio-Rad Laboratory, Inc., CA) as follows: after a 5 min denaturation at 95°C, the reaction was run for 40 cycles, each comprising 45 s of denaturing at 95°C, 45 s of annealing at variable temperatures according to the primers, and 45 s of extension at 72°C, with an extension at 72°C for 5 min as the last step. The PCR products were electrophoresed on a 1.2% agarose gel and visualized under UV illumination using an ethidium bromide stain.

A biotin-labeled primer was used in the PCR in order to purify the final product from the mix by using streptavidin-coated Sepharose beads (10058558; GE Healthcare, Milwaukee, WI). Sequencing primer was then annealed to the purified single-stranded PCR product, and pyrosequencing was performed on a PyroMarkQ24 System (Qiagen, Germany) according to manufacturer's instructions. To provide an internal control for total bisulfite conversion, a non-CG

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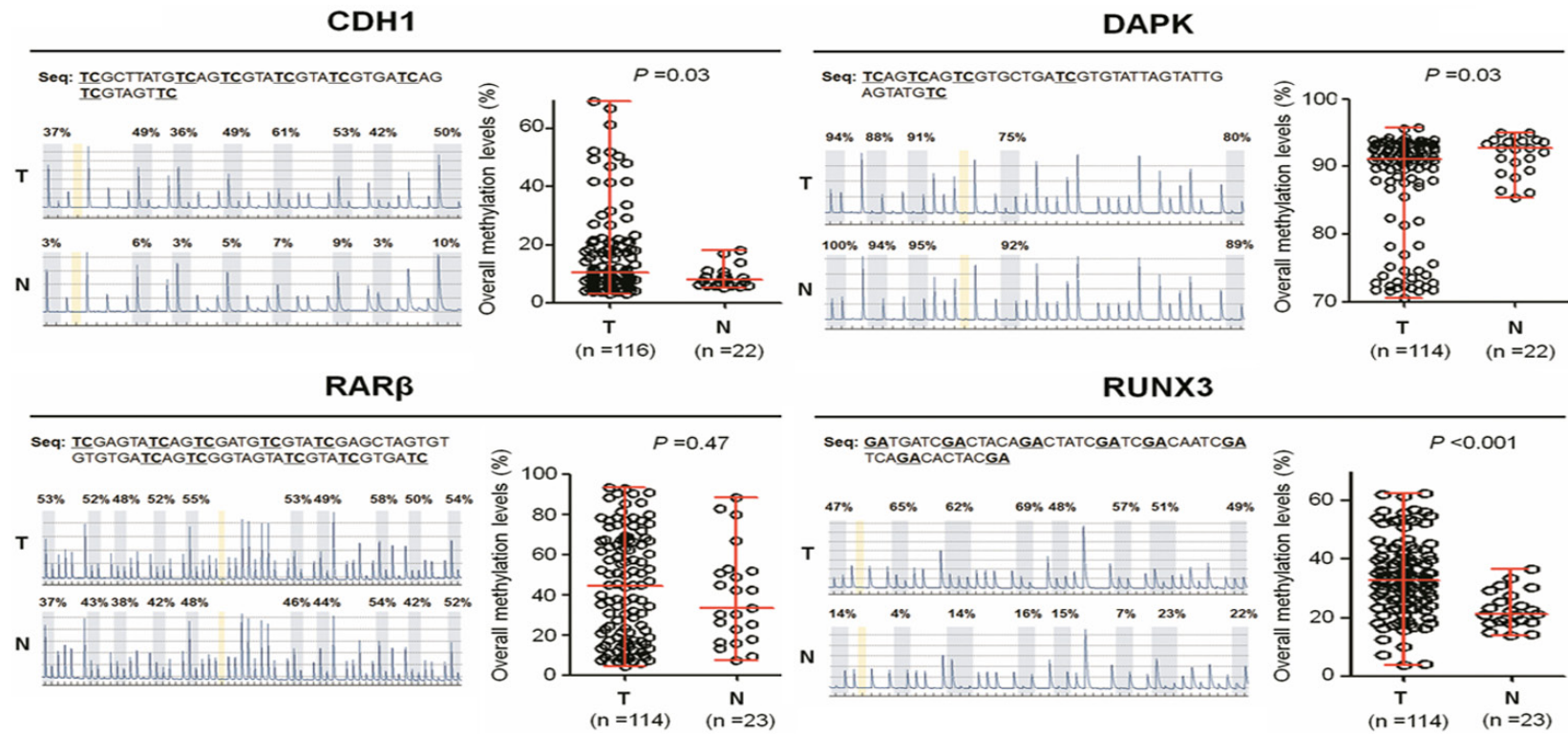


Figure 1. Analysis of overall methylation levels of four tumor suppressor genes in PTC samples using pyrosequencing assay. Pyrosequencing method was performed as described in Methods. Representative pyrograms show methylation levels of the indicated CpG sites in gene promoter (left panel). The average methylation level of each gene is represented in the right panel. Horizontal lines represent the median and the 25th to 75th percentile. T, PTC tissues; N, goiter tissues. *P* values < 0.05 were considered significant.

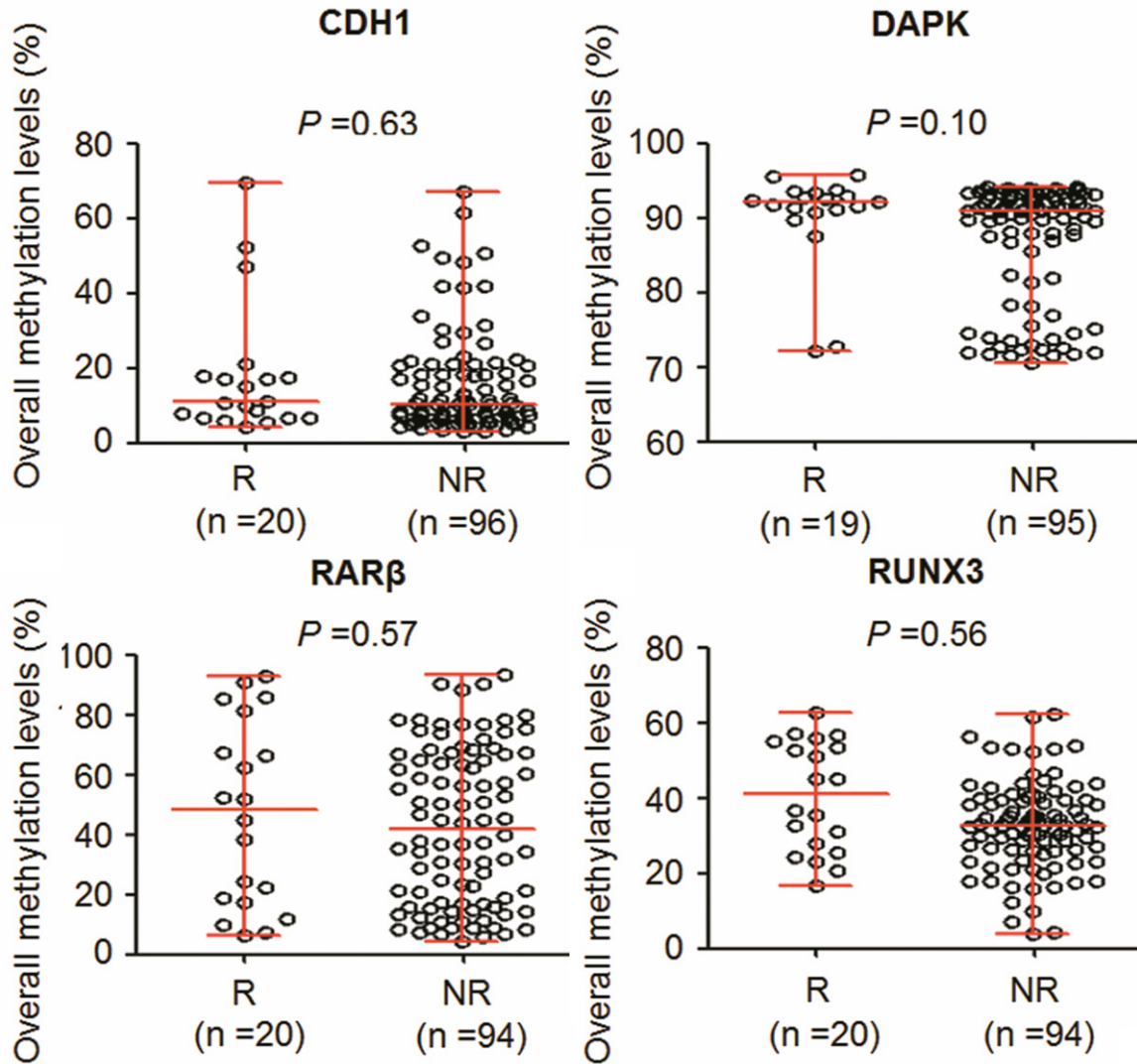


Figure 2. Association of overall methylation levels of these four genes with tumor recurrence. Shown were scatterplots of average methylation levels of each gene in recurrent (R) and non-recurrent (NR) cases. Horizontal lines represent the median and the 25th to 75th percentile.

cytosine was included in the region targeted for pyrosequencing where possible. Target CpG sites were evaluated by using the instrument software (PSQ24MA 2.0.6, Qiagen), which converts pyrograms to numerical values for peak heights and calculates the proportion of methylation at each base as a C/T ratio.

Detection of *BRAF*^{V600E} mutation by pyrosequencing

A 228-bp fragment from exon 15 of the *BRAF* gene spanning the hotspot mutation site at codon 600 was analyzed by pyrosequencing method as described previously [16].

Statistical analysis

The output data include site-specific percent methylation for each CpG in the investigated regions as well as overall average percent methylation, which is the average of the CpG methylation levels within the promoter. Overall or site-specific methylation levels of each gene between recurrent and non-recurrent groups were compared using the independent sample *t*-test or non-parametric Wilcoxon's Rank Sum test according to the distribution type of the data. Similar statistical strategy was used to evaluate the association of *BRAF* mutation with tumor recurrence. To predict disease recur-

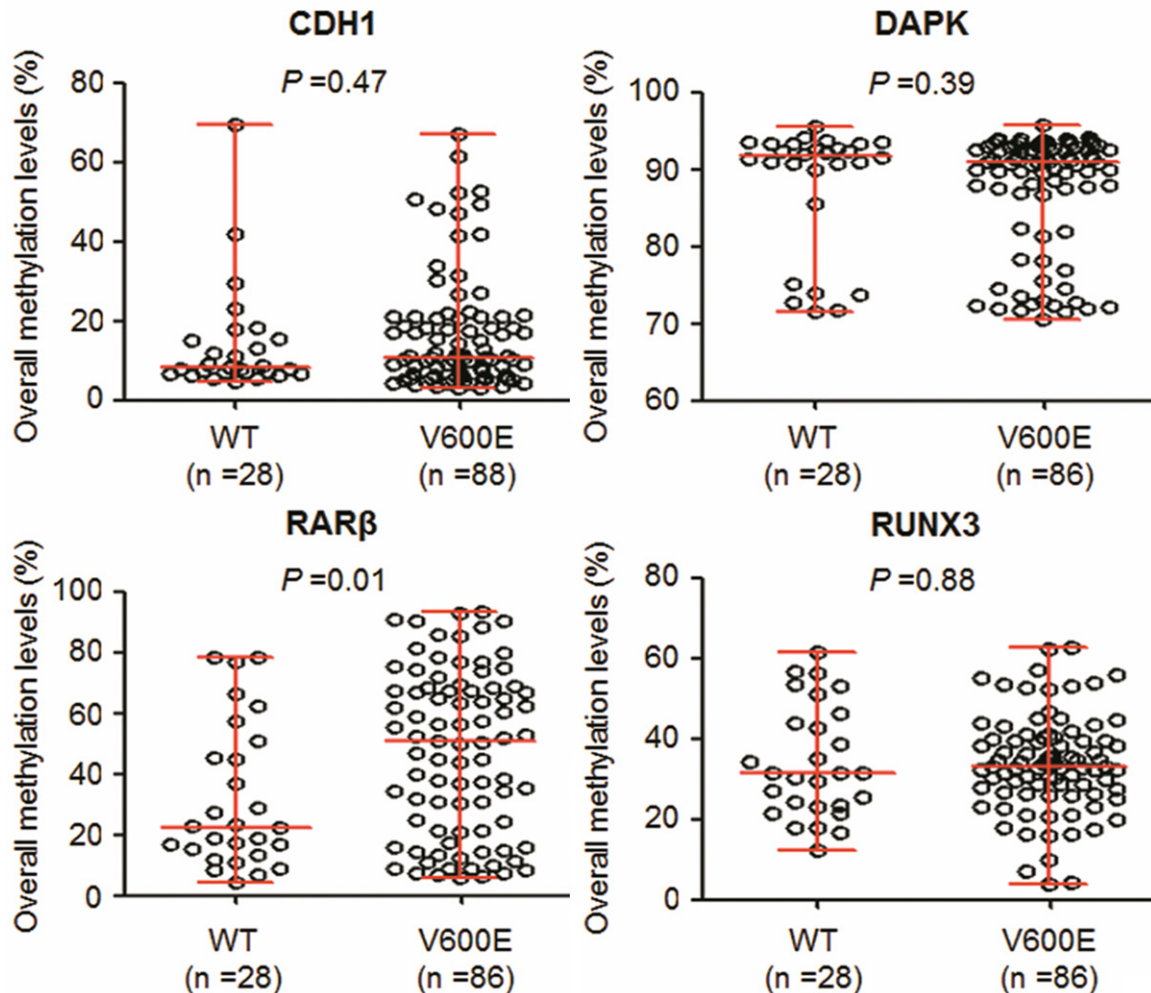


Figure 3. Association of *BRAF* mutation with overall methylation levels of these four genes in PTCs. Shown were scatterplots of overall methylation levels of each gene in *BRAF* mutation-negative (WT) and -positive (V600E) cases. Horizontal lines represent the median and the 25th to 75th percentile. *P* values < 0.05 were considered significant.

rence, we set up site-specific cut-off values by constructing receiver operator characteristic (ROC) curves using MedCalc Software (MedCalc Software bvba, Belgium). Site-specific sensitivity and specificity, positive and negative predictive values were calculated using standard formulas. Univariate and multivariable logistic regression analyses were performed to estimate the odds ratios (ORs) of clinicopathologic characteristics and disease recurrence according to site-specific methylation levels, independent of gender, age, lymph node metastasis and TNM stage. Kaplan-Meier survival analysis and standard log-rank test were used to evaluate the effect of site-specific methylation levels on tumor recurrence. All statistical analyses were performed using the SPSS statistical

package (11.5, Chicago, IL, USA). *P* values < 0.05 were considered significant.

Results

Overall average methylation levels of CDH1, DAPK, RUNX3 and RARβ genes in PTCs

We evaluated promoter methylation of *CDH1*, *DAPK*, *RUNX3* and *RARβ* genes in 120 PTCs and 23 goiter tissues by using pyrosequencing method. As shown in **Figure 1**, overall average methylation levels of *CDH1*, *RUNX3* and *RARβ* were higher in PTCs than in control subjects. In contrast, overall average methylation levels of *DAPK* were lower in PTCs than in control subjects. Among these genes examined, statistical significances were found in *CDH1* (*P* = 0.03),

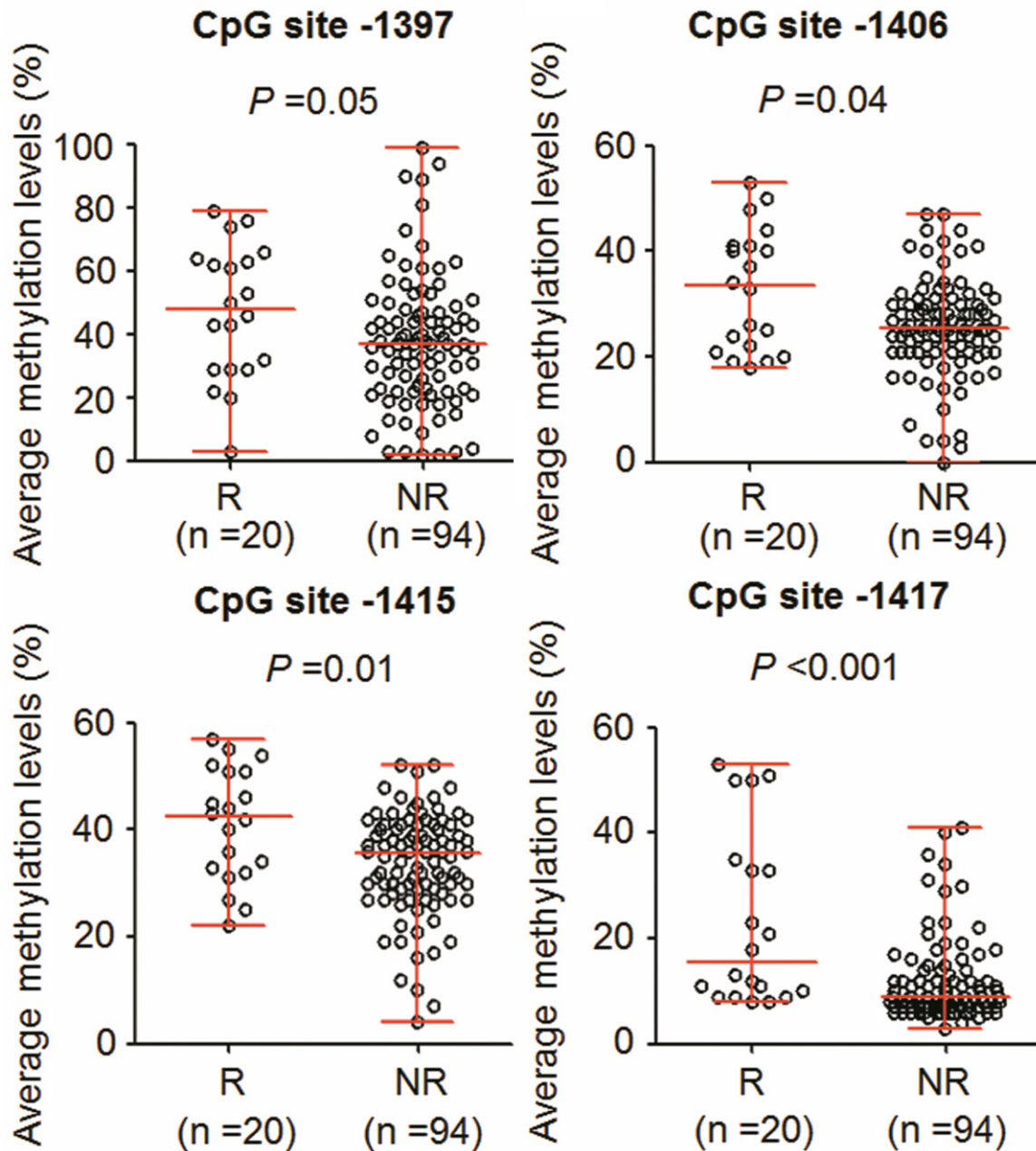


Figure 4. Association of *RUNX3* site-specific methylation levels with tumor recurrence. Shown were scatterplots of *RUNX3* site-specific methylation levels in recurrent (R) and non-recurrent (NR) cases. Horizontal lines represent the median and the 25th to 75th percentile. *P* values < 0.05 were considered significant.

DAPK ($P = 0.03$), and *RUNX3* ($P < 0.001$). Given the importance of promoter methylation in thyroid tumorigenesis, the association of promoter methylation of these genes with clinicopathological features, including gender, age, lymph node metastasis, TNM stage and tumor recurrence, was explored in a cohort of PTCs. However, our data showed that overall average methylation levels of these four genes did not significantly correlate with any clinicopathological variables in PTCs (data not shown). Although

overall average methylation levels of these four genes were a little bit higher in recurrent cases than in non-recurrent cases, the differences did not reach statistical significance (**Figure 2**).

Detection of BRAF^{V600E} mutation in PTCs using pyrosequencing

We used pyrosequencing assay to test *BRAF^{V600E}* mutation in a total of 120 PTCs. In these PTCs, 92 of 120 (76.7%) cases were positive

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Table 1. The values of *RUNX3* site-specific hypermethylation in predicting PTC recurrence

CpG sites	AUC ¹	Cut-off values	OR (95% CI) ²	P values	Sensitivity (%)	Specificity (%)	PPV (%) ³	NPV (%) ⁴
-1397	0.64	59.0%	4.50 (1.53-13.26)	0.011	40.0 (8/20)	87.1 (81/93)	40.0 (8/20)	87.1 (81/93)
-1406	0.65	40.5%	5.33 (1.56-18.18)	0.013	30.0 (6/20)	92.6 (87/94)	46.2 (6/13)	86.1 (87/101)
-1415	0.69	42.5%	5.71 (2.01-16.24)	0.001	50.0 (10/20)	85.1 (80/94)	41.7 (10/24)	88.9 (80/90)
-1417	0.75	32.0%	12.12 (3.11-47.16)	0.000	35.0 (7/20)	95.7 (90/94)	63.6 (7/11)	87.4 (90/103)

¹AUC (area under the curve). ²OR: odds ratio with 95% confidence interval (CI). ³PPV, positive predictive value. ⁴NPV, negative predictive value.

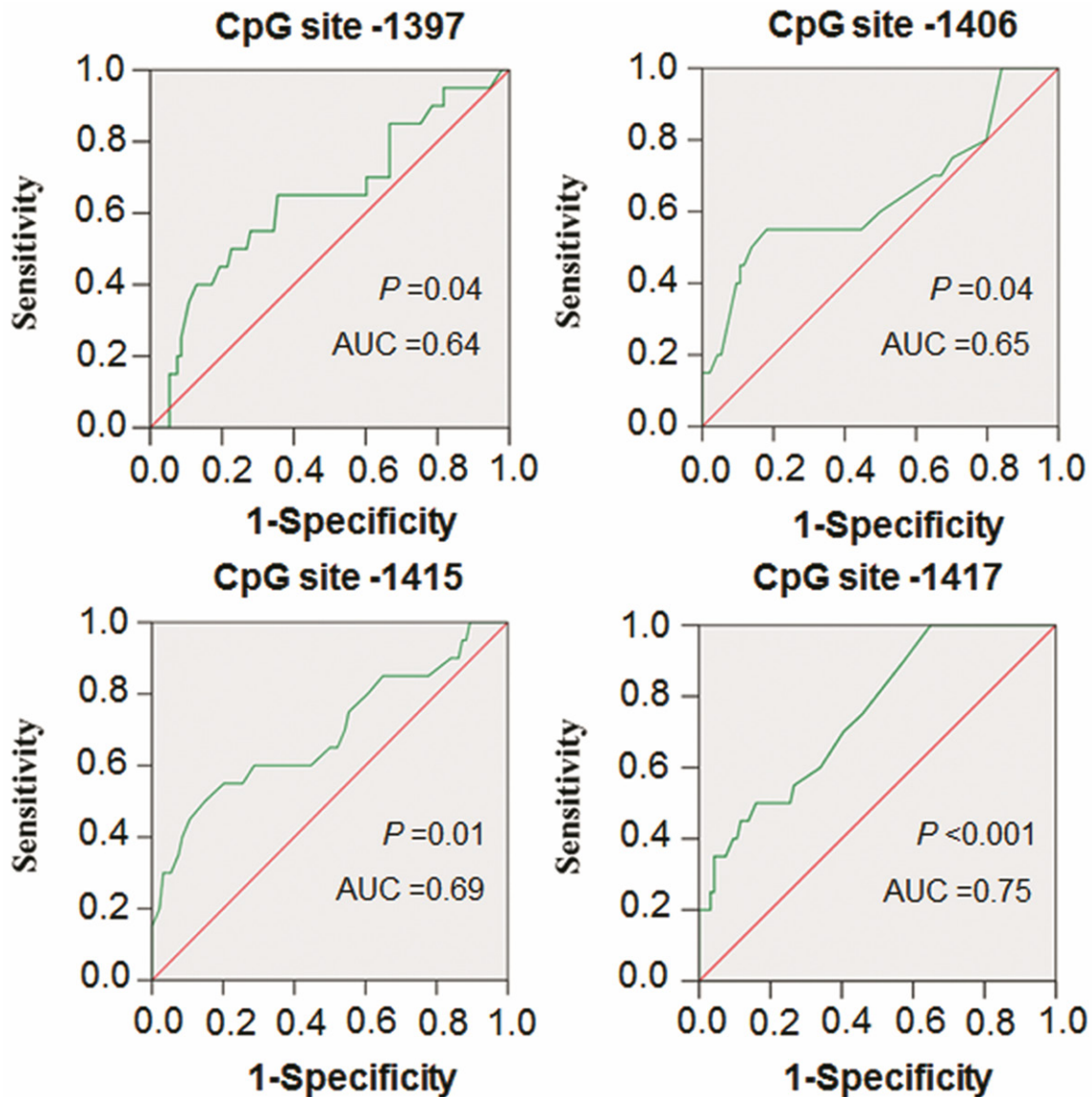


Figure 5. Receiver operating characteristic (ROC) curves for four CpG sites in the *RUNX3* promoter. All recurrent and non-recurrent PTCs for which there was complete DNA methylation data were used for the analysis. The ROC curves plot sensitivity and 1-specificity. Areas under the curve (AUC) and P values were shown in the graph.

for *BRAF* mutation. As expected, all control subjects were negative for *BRAF* mutation. Given that previous studies have revealed an association of *BRAF*^{V600E} mutation with promoter methylation of tumor suppressor genes in thyroid

cancer [16-20], we thus tested the association of methylation levels of these genes with this mutation found in these PTCs. Of these four methylation markers investigated, *BRAF* mutation was only positively associated with overall

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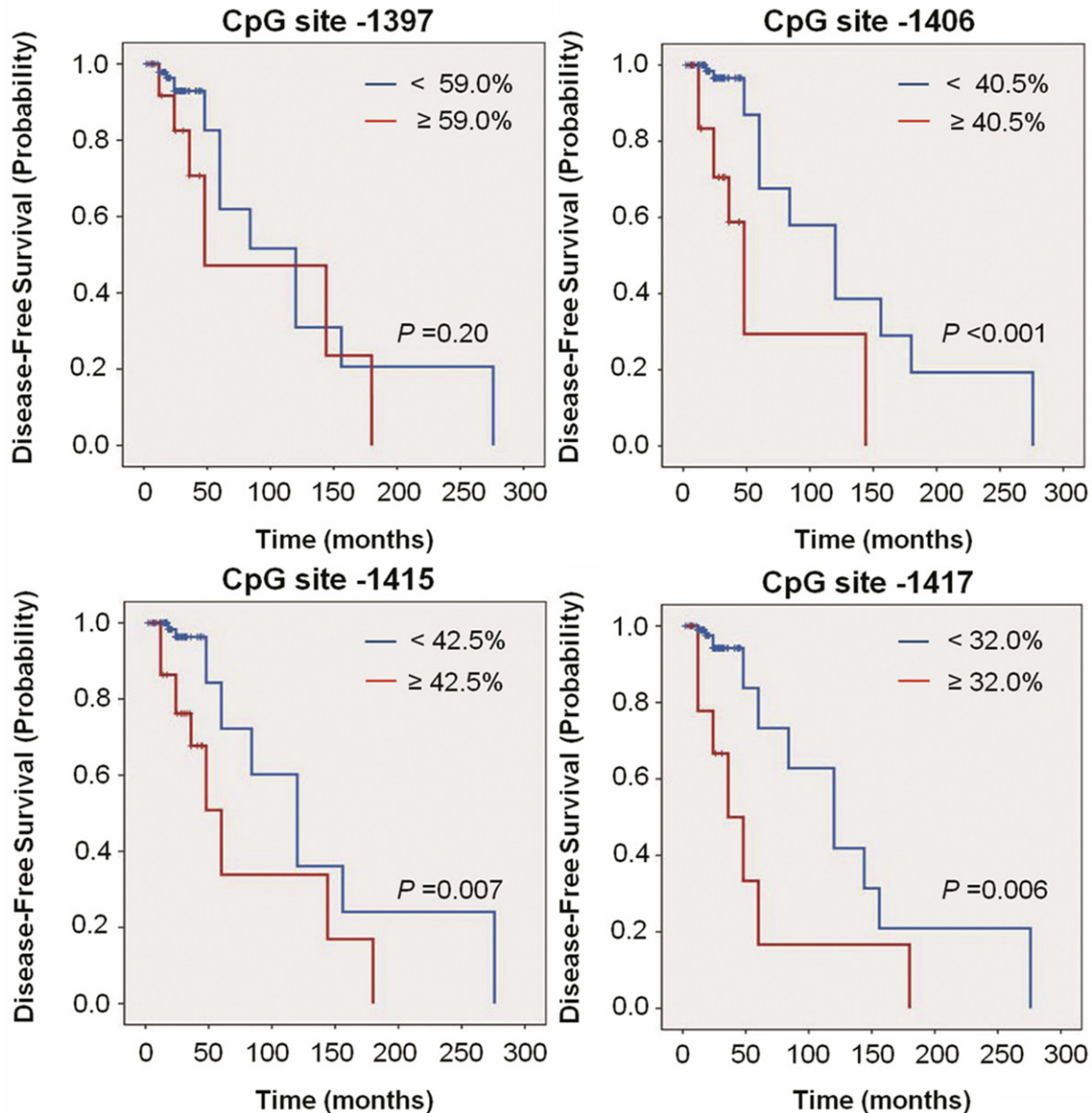


Figure 6. Kaplan-Meier estimate of PTC disease-free probability in patients with different *RUNX3* site-specific methylation levels. Short vertical lines indicate censored observations (months of follow-up for those who have no had recurrence/persistence).

average methylation levels of *RARβ* ($P = 0.01$) by Wilcoxon test analysis (Figure 3). In addition, we investigated the relationship between *BRAF* mutation and disease recurrence. The results showed that this mutation was not significantly associated with tumor recurrence ($P = 0.629$).

Association of RUNX3 site-specific methylation with tumor recurrence

The site-specific percent methylation of each of multiple CpG sites within *CDH1*, *DAPK*, *RUNX3*

and *RARβ* promoters was measured and investigated their association with clinicopathologic characteristics in PTCs. The results showed that site-specific methylation levels in these gene promoters did not significantly correlate with most of clinicopathologic variables (data not shown). However, we found that methylation levels of *RUNX3* at CpG sites -1397, -1406, -1415 and -1417 were associated with tumor recurrence. Methylation levels at these CpG sites were much higher in recurrent cases than in non-recurrent cases, particularly at the latter

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Table 2. The prognostic value of clinicopathological factors and *RUNX3* site-specific hypermethylation in multivariate Cox regression analysis

Variables	<i>RUNX3</i> gene							
	CpG site -1397		CpG site -1406		CpG site -1415		CpG site -1417	
	Hazard Ratio (HR) (95% CI)	<i>P</i>	Hazard Ratio (HR) (95% CI)	<i>P</i>	Hazard Ratio (HR) (95% CI)	<i>P</i>	Hazard Ratio (HR) (95% CI)	<i>P</i>
Methylation levels								
< cut-off values	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
≥ cut-off values	5.83 (1.66-20.44)	0.006	16.92 (4.42-64.76)	0.000	8.06 (2.47-26.27)	0.001	12.69 (2.94-54.69)	0.001
Gender								
Female	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
Male	0.53 (0.13-2.26)	0.39	0.94 (0.24-3.72)	0.93	0.75 (0.20-2.85)	0.67	1.15 (0.29-4.57)	0.85
Age								
≤ 40	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
> 40	0.11 (0.01-0.93)	0.04	0.26 (0.04-1.84)	0.18	0.21 (0.03-1.56)	0.13	0.27 (0.03-2.23)	0.23
Lymph node metastasis								
No	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
Yes	2.72 (0.66-11.24)	0.17	4.60 (1.08-19.72)	0.04	3.81 (0.91-16.00)	0.07	3.61 (0.96-13.68)	0.06
TNM Stage								
< III	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
≥ III	5.11 (0.75-34.93)	0.09	1.82 (0.34-9.61)	0.48	1.67 (0.31-9.07)	0.55	0.71 (0.10-4.95)	0.73

three sites ($P = 0.04$ at CpG site -1406; $P = 0.01$ at CpG site -1415; $P < 0.001$ at CpG site -1417) (Figure 4).

Next, we set up appropriate cut-off values for these four CpG sites to accurately predict tumor recurrence by employing receiver operator characteristic (ROC) curves. ROC curve is graphic presentation of the relationship between both sensitivity and specificity and it helps to decide the optimal model through determining the best threshold for a diagnostic/predictive test [21]. The area under the curve (AUC) of the ROC curves provides a way to measure the accuracy of a diagnostic/predictive test. The large area, the more accurate the test is [21]. As shown in Table 1 and Figure 5, all these four CpG sites showed high AUC of the ROC curves, ranging from 0.64 to 0.75. Importantly, *RUNX3* site-specific hypermethylation was associated with a significantly increased risk of tumor recurrence (CpG site -1397: OR = 4.50, 95% CI = 1.53-13.26, $P = 0.011$; CpG site -1406: OR = 5.33, 95% CI = 1.56-18.18, $P = 0.013$; CpG site -1415: OR = 5.71, 95% CI = 2.01-16.24, $P = 0.001$; CpG site -1417: OR = 12.12, 95% CI = 3.11-47.16, $P = 0.000$). These findings suggest that *RUNX3* site-specific hypermethylation may be a dangerous factor for PTC recurrence. To evaluate the value of *RUNX3* site-specific methylation in predicting PTC recurrence, we calculated their sensitivity and specificity by the use

of the same cut-off values. Also shown in Table 1, although the sensitivities of these four CpG sites were not too high, ranging 30.0 to 50.0%, they showed the excellent specificities, ranging from 85.1 to 95.7%, and the negative predictive values of 86.1 to 88.9%. Taken together, *RUNX3* site-specific hypermethylation has predictive value for postoperative recurrence of PTC patients.

The prognostic value of *RUNX3* site-specific hypermethylation for PTC recurrence was further demonstrated as dramatically reduced disease-free probability by Kaplan-Meier analysis using the same cut-off values (CpG -1397: 95.1 months vs. 125.2 months on average, $P = 0.20$; CpG -1406: 65.7 months vs. 134.0 months on average, $P < 0.001$; CpG -1415: 80.3 months vs. 138.1 months on average, $P = 0.007$; CpG -1417: 59.3 months vs. 135.6 months on average, $P = 0.006$) (Figure 6). We next want to explore whether the prognostic value of these site-specific hypermethylation is attributable to its relationship with other potential clinicopathologic confounding factors, including gender, age, lymph node metastasis and TNM stage, or whether they contribute independently to prognosis. Thus, the independent relationship of these site-specific hypermethylation with disease-free survival was studied using Cox multivariate regression analysis. The results indicated that *RUNX3* site-spe-

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cific hypermethylation is a predictor of poor prognosis for PTC recurrence as an independently variable with respect to gender, age, lymph node metastasis and TNM stage (Table 2).

Discussion

It is well known that patients with PTC have excellent prognosis, but 5%-40% of cases have persistent or recurrent disease even after curative surgery [22, 23]. Although radioiodine treatment may reduce recurrence, ¹³¹I ablation was effective in only 10% of high-risk PTC patients according to the AMES (age, metastasis to distant sites, extrathyroidal invasion, and size) classification, and was not effective in 30% of low-risk PTC patients [24-26]. Thus, early predicting the risk of cancer recurrence, such as aggressive PTCs, is very important to improve clinical outcomes of patients.

Assays of serum thyroglobulin (Tg) concentrations combined with high-resolution ultrasound have been widely used for postoperative follow-up, enabling early detection of recurrence and more prompt management [27, 28]. However, the utility of Tg testing is limited by autoantibody interference in 25% of patients [29, 30], and the accuracy of ultrasonography has been found to vary widely, from 40% to 90%, and to be operator-dependent [31]. In recent years, a number of evidences have demonstrated the association of gene methylation with disease recurrence in diverse cancers [14, 32-34], including thyroid cancer [35]. However, these studies make use of methods that qualitatively analyze particular CpG sites within gene promoter or quantitatively analyze overall methylation levels of candidate gene as well as high-cost and global screening of aberrant DNA methylation. Therefore, in this study, we attempted to use pyrosequencing method to afford a more detailed survey of methylation state and better assess the recurrence risk of postoperative PTC patients. There are several reasons to support the point that the use of pyrosequencing assay to quantitatively analyze multiple CpG sites with gene promoter may be a useful and cost-effective strategy for predicting PTC recurrence. First, promoter methylation by gene silencing of tumor-related genes, particularly tumor suppressor genes, is one of the commonest molecular events in human tumorigenesis and progression, including PTC [5, 8,

36]. Second, pyrosequencing is one of the most accurate methods available for quantifying DNA methylation at specific CpG sites within the target region of interest. It is a sensitive and highly reproducible method that is uniquely suited to the analysis of a small fraction of the total DNA in the clinical samples [37, 38].

In this study, we used pyrosequencing method to analyze *BRAF*^{V600E} mutation and promoter methylation of four tumor suppressor genes in a cohort of PTCs and explore their association with PTC recurrence. Our data showed that the prevalence of *BRAF* mutation was 76.7%, and was highly specific for PTCs. However, *BRAF* mutation was not associated with PTC recurrence, which was consistent with a previous study [7]. Methylation analysis showed that two of these four genes tested had significantly higher overall methylation levels in PTCs than in control subjects, including *CDH1* and *RUNX3* genes. *CDH1* (also known as E-cadherin) is a transmembrane glycoprotein that is localized in the adherens junctions of epithelial cells [39]. *CDH1* inactivation by promoter methylation is thought to contribute to tumor progression through increased proliferation, invasion and metastasis [39, 40]. *RUNX3* belongs to the *RUNX* family of transcription factor and acts as strong tumor suppressor activity through modulating a series of cancer-associated genes, such as p53, p21, Notch1 and p27, which is involved in the regulation of epithelial proliferation and apoptosis [41]. DNA methylation-mediated *RUNX3* inactivation has also been reported in various solid tumors, including PTC and plays a critical role in PTC tumorigenesis [42].

Next, we sought to explore the association of methylation levels of these genes with PTC recurrence. Unfortunately, our data suggested that overall methylation levels of these genes did not significantly correlate with disease recurrence. However, when we further analyzed the association of CpG site-specific methylation levels in each promoter with PTC recurrence, we found that high methylation levels of *RUNX3* at CpG sites -1397, -1406, -1415 and -1417 were associated with a significantly increased risk of disease recurrence. Moreover, multivariable logistic regression showed that *RUNX3* site-specific hypermethylation was a significant independent predictor for PTC recurrence. Importantly, using these CpG sites with appropriate cut-off values, we were able to

obtain the excellent specificities and the negative predictive values.

In summary, in this study, we investigated methylation levels of *CDH1*, *DAPK*, *RARβ* and *RUNX3* genes and *BRAF*^{V600E} mutation in a cohort of PTCs using pyrosequencing method and demonstrated the association of *RUNX3* site-specific methylation levels with PTC recurrence. These observations provide strong evidences suggesting that *RUNX3* site-specific hypermethylation may be potential and reliable biomarkers to predict or monitor postoperative recurrence of PTC patients.

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

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Supplementary Table 1. Clinicopathological characteristics of PTC patients

Characteristics	No. of patients (%)
Gender	
Male	30 (25.00%)
Female	90 (75.00%)
Age (years)	
≤ 40	62 (51.67%)
> 40	58 (48.33%)
Lymph node metastasis	
Yes	60 (50.00%)
No	60 (50.00%)
TNM Stage	
< III	87 (72.50%)
≥ III	33 (27.50%)
Recurrence	
Yes	20 (16.67%)
No	100 (83.33%)

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Supplementary Table 2. Primer sequences used in this study

Genes	Forward primers (5'→3')	Reverse primers (5'→3')	Sequencing primers (5'→3')	Product length (bp)	No. of CpG sites	Annealing temperature (°C)
<i>CDH1</i>	ATTTGTGAGTTTGAGGAAGTTAGTTTAGA	Biotin-ACTCCAAAAACCATAACTAACC	TTTGAGGAAGTTAGTTTAGATTTTA	117	8	57
<i>DAPK</i>	TGGATATGGGATTTTGTGTAGA	Biotin-ACCAAATAATCTCCATCTTTAAC	TTTTAAAAAGTAAATAGGTGA	201	5	58
<i>RARB</i>	ATTTTTGTAAAGGGGGGATTAG	Biotin-TTCCCAAAAAATCCCAAATTCTCCTT	GTTGTTTGAGGATTGG	181	10	59
<i>RUNX3</i>	Biotin-AAGGGGTGATTTGTAGTGAAGTTTA	CTCTACCAATCCAACCCCACTTCTTCT	CCCACTTCTTCTTAAACC	169	8	60