

# Aberrant hypermethylation of the *HOXD10* gene in papillary thyroid cancer with *BRAF*<sup>V600E</sup> mutation

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**Abstract.** Epigenetic abnormalities as well as genetic abnormalities may play a vital role in the tumorigenesis of papillary thyroid cancer (PTC). The present study aimed to analyze the function and methylation status of the *HOXD10* gene in PTC and aimed to identify relationships between *HOXD10* methylation, *HOXD10* expression, *BRAF* mutation and clinicopathological characteristics of PTC. A total of 152 PTC patients were enrolled in the present study. The methylation status of the *HOXD10* promoter was analyzed by quantitative methylation-specific polymerase chain reaction (Q-MSP). *BRAF*<sup>V600E</sup> mutation status was analyzed by polymerase chain reaction (PCR) followed by DNA sequencing. *HOXD10* mRNA expression level was analyzed by real-time polymerase chain reaction (RT-PCR). 5-Aza-2-deoxycytidine (5-Aza) treatment was performed in 4 PTC cell lines to observe the change in *HOXD10* expression. Transwell, cell cycle and apoptosis assays were then performed in an *HOXD10*-overexpressing PTC cell line. Furthermore, we analyzed the associations between *HOXD10* methylation, *HOXD10*

expression, *BRAF* mutation and clinicopathological characteristics in PTC. Overexpression of *HOXD10* suppressed the migration of PTC cells, and promoted cell apoptosis. Q-MSP showed that methylation levels of the *HOXD10* promoter were significantly higher in PTC tissues than levels in the adjacent normal thyroid tissues (P=0.02). In addition, expression of *HOXD10* was decreased in the PTC cell lines and PTC tissues compared with that noted in the adjacent normal thyroid tissues (P=0.008). However, *BRAF*<sup>V600E</sup> mutation was detected in 42.1% of PTC patients enrolled. In addition, the *BRAF* mutation status was associated with the methylation and expression level of *HOXD10* in PTC. We then observed that 5-Aza treatment could revert the expression of *HOXD10* in PTC cell lines. Moreover, the hypermethylation of *HOXD10* was associated with invasion of the primary tumor and age >45. In conclusion, the *HOXD10* gene may act as a tumor suppressor in PTC. The aberrant hypermethylation and decreased expression of the *HOXD10* gene were shown in PTC patients, particularly in those with *BRAF*<sup>V600E</sup> mutation. The epigenetic suppression of the *HOXD10* gene may play a role in the tumorigenesis of PTC, and it is a prospective biomarker for the diagnosis and prognosis of PTC.

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**Key words:** papillary thyroid cancer, methylation, *HOXD10*, *BRAF* mutation

## Introduction

Thyroid cancer is the most prevalent endocrine malignancy in humans. The incidence of thyroid cancer has increased in recent years. Papillary thyroid cancer (PTC) is a major type (80-85%) of thyroid cancer (1). Along with the improvement of diagnostic approaches for thyroid cancer, more and more PTC cases have been diagnosed (2). Although the prognosis of most PTC patients is optimistic, the recurrence rate was found to be relatively high after a 15-year follow-up (3-5). A small group of PTC patients appear to have a higher risk of recurrence and metastasis (6). To distinguish the population of patients with higher risk is an important task in the clinic. Appropriate biomarkers could be used to help evaluate the

recurrence and metastasis risk of PTC. However, there are no effective biomarkers currently used in the clinic. New molecular biomarkers are urgently needed for the identification of the PTC patients with higher recurrence and metastasis risk.

Genetic research, which has been used as a molecular method for genetic discovery, has played a vital role in understanding the process of tumorigenesis. *BRAF*<sup>V600E</sup> mutation, by far, is the most common genetic event found in PTC, occurring in 20-50% of cases (7-11). Yet, PTC tumorigenesis may be regulated by epigenetic events as well. DNA methylation is the most common epigenetic regulatory mechanism in tumorigenesis. Evaluating the methylation status of DNA could be useful for the diagnosis, prognostic evaluation and predicting the risk for recurrence and metastasis of PTC (12-15).

In previous studies, we performed methylated DNA immunoprecipitation sequencing (MethylCap-seq) assay and established a database of the genome-wide DNA methylation profile of PTC. *HOXD10* was one of the candidate genes that were aberrantly hypermethylated in PTC (Fig. 1). In the present study, we aimed to analyze the function and methylation status of the *HOXD10* gene in PTC and to elucidate the relationship between *HOXD10* methylation, *HOXD10* expression, *BRAF* mutation and clinicopathological characteristics.

## Materials and methods

**Clinical samples.** Human primary PTCs and adjacent non-tumor tissues (2 cm away from the tumor edge) were collected from patients who were initially surgically treated at the Department of Head and Neck Surgery, Fudan University Shanghai Cancer Center (Shanghai, China). All the patients had received lobectomy and isthmectomy plus ipsilateral central lymph node dissection. Additional modified lateral lymph node dissection was performed in patients with clinically suspicious lateral lymph node metastasis. All of the samples were pathologically confirmed. Totally 152 PTC patients were enrolled from April 2014 to December 2014. All the samples were stored at -80°C. Informed consent for the use of the tissues for clinical research was obtained before surgery, and the study protocol and consent form were approved by the Ethics Committee of Fudan University Shanghai Cancer Center. The tumor-node-metastasis (TNM) stages were determined according to the American Joint Cancer Committee (AJCC) TNM grading system, 7th edition. The clinicopathological data of the patients enrolled are summarized in Table I.

**Cell culture and 5-Aza-2-deoxycytidine treatment.** Human PTC cell lines TPC-1, BCPAP, K1, W3 were used for the present study (16). The cell lines TPC-1 and BCPAP were routinely cultured at 37°C in RPMI-1640 medium with 10% fetal bovine serum (FBS). K1 and W3 cells were cultured in DMEM/Hams F-12 medium (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA). All the media were supplemented with penicillin/streptomycin. In some experiments, tumor cells were treated with 5  $\mu$ M/ml 5-Aza-2'-deoxycytidine (5-Aza) for 72 h as a demethylation treatment. Media and 5-Aza were replenished every 24 h.

**Plasmid construction and cell transfection.** The *HOXD10*-overexpressing plasmid was constructed by cloning of the

Table I. Clinical characteristics of the PTC patients.

Characteristic	Data
Patients, n	152
Age (years)	
Mean	42.72 $\pm$ 13.309
>45	69 (45.4)
$\leq$ 45	83 (54.6)
Sex, n (%)	
Male	33 (21.7)
Female	119 (78.3)
Invasion, n (%)	
Yes	29 (19.1)
No	123 (80.9)
Size (cm)	
Mean	1.419 $\pm$ 0.849
>1	94 (61.8)
$\leq$ 1	56 (36.8)
Multifocality, n (%)	
Yes	50 (32.9)
No	102 (67.1)
Bilaterality, n (%)	
Yes	30 (19.7)
No	121 (79.6)
Hashimoto's thyroiditis, n (%)	
Yes	29 (19.1)
No	123 (80.9)
Surgery, n (%)	
Lobectomy + isthmectomy + CLND	111 (73.0)
Lobectomy + isthmectomy + CLND + LLND	41 (27.0)
Central lymph node metastasis, n (%)	
Yes	79 (52.0)
No	73 (48.0)
Lateral lymph node metastasis, n (%)	
Yes	41 (27.0)
No	111 (73.0)

Data are presented as n (%) or mean  $\pm$  standard deviation. CLND, central lymph node dissection; LLND, lateral lymph node dissection.

full-length *HOXD10* open reading frame into the mammalian expression vector pcDNA3.1 with *Bam*HI and *Xho*I restriction enzyme sites. The sequences were confirmed by DNA sequencing. PTC TPC-1 cells were cultured into a 6-well plate for 24 h and transfected with pcDNA3.1-*HOXD10* or empty vector pcDNA3.1 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After confirming the transfection efficiency by RT-PCR and western blotting in the surviving colonies, cells were transferred into a 6-well plate, and cultivated for further use.

**Cell migration assay.** PTC cells transfected with pcDNA3.1 vector or pcDNA3.1-*HOXD10* were used for cell migration

Table II. Primer sequences for RT-PCR, Q-MSP and *BRAF* sequencing.

Reaction	Primer sequences (5'-3')
<i>HOXD10</i> -RT-F	CTGAGGTCTCCGTGTCCAGT
<i>HOXD10</i> -RT-R	CTGAGGTCTCCGTGTCCAGT
GAPDH-RT-F	GGCCTCCAAGGAGTAAGACC
GAPDH-RT-R	CAAGGGGTCTACATGGCAAC
<i>HOXD10</i> -Q-MSP-F	TGGAGAGGCGGACAGGAG
<i>HOXD10</i> -Q-MSP-R	GGGTAAGCACGGACAACAGAGC
<i>HOXD10</i> -Q-MSP-probe	6FAM-CCAGCGCGCACTATCGCGG-TAMRA
<i>ALU</i> -Q-MSP-F	GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA
<i>ALU</i> -Q-MSP-R	ATTAATAAATAATCTTAACTCCTAACCTCA
<i>ALU</i> -Q-MSP-probe	6FAM-CCTACCTTAACCTCCC-MGB
<i>BRAF</i> V600E-F	CATAATGCTTGCTCTGATAGGAAAATG
<i>BRAF</i> V600E-R	CTGATGGGACCCACTCCAT

RT-PCR, real-time polymerase chain reaction; Q-MSP, quantitative methylation-specific polymerase chain reaction; F, forward; R, reverse.

assays. Cell migration was assessed by modified Boyden Transwell chambers assay (Corning, Corning, NY, USA). Briefly,  $2 \times 10^4$  cells/well were plated into 100  $\mu$ l of no FBS medium in the upper chamber, and 500  $\mu$ l of medium containing 10% FBS was added to the lower chamber. The cells were incubated for 12 h. The nonmigratory cells in the upper chamber were removed with a cotton swab. The cells on the bottom of the membrane were fixed and stained with poly-fluoroalkoxy (PFA) and crystal violet stain solution (0.5%). The number of visible cells was counted by fluorescence microscope in 5 random high power fields. All the experiments were repeated 3 times.

**Cell apoptosis and cell cycle.** Analysis of cell apoptosis was performed using the PE Annexin V apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) by flow cytometric analysis (FCA). Briefly, stably transfected PTC cell line TPC-1 was suspended in annexin binding buffer, Alexa Fluor 488 Annexin V and propidium iodide (PI) working solution were added in sequence. The stained cells were finally analyzed by FACScan flow cytometry (BD Biosciences). Cell cycle distribution was detected by the Cycletest™ Plus DNA Reagent kit (BD Biosciences). Briefly, transfected cells were harvested and washed in PBS. Cellular DNA was stained with 125  $\mu$ g/ml PI for 20 min at 4°C in dark. The cells were then sorted by FACSCalibur, and cell cycle distribution was determined using the ModFit LT software (Verity Software House, Topsham, ME, USA).

**Western blot analysis.** Total proteins were extracted from the stably transfected cells using RIPA lysis buffer. Lysates were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Primary antibodies were used as follows: *HOXD10* (1:1,000; Abcam, Cambridge, MA, USA) and *Tubulin* (1:1,000;

Proteintech Group, Chicago, IL, USA). The blots were developed using chemiluminescence with Las 4000 imaging system (Fujifilm, Tokyo, Japan).

**DNA extraction and bisulphite conversion.** Fresh-frozen tissue specimens and PTC cell lines were homogenized using a bead homogenizer and genomic DNA was extracted using the Genomic DNA Extraction kit (Tiangen, Beijing, China) according to the manufacturer. The DNA concentration was determined by NanoDrop 1000. The bisulfite conversion is described by Yu *et al* (17). The DNA sample was then stored at -20°C until further use.

**Quantitative methylation-specific PCR (Q-MSP).** Q-MSP assay was performed to analyze the methylation level of the *HOXD10* gene in PTC. We used a plasmid vector to construct a standard curve for absolute quantification PCR. The repetitive DNA element *ALU* was used as an internal reference. Briefly, quantitative PCR was carried out in a final reaction mixture of 20  $\mu$ l containing 4  $\mu$ l bisulfite-treated DNA, 500 nM of each primer, 250 nM TaqMan probe, 1.875 mM MgCl<sub>2</sub>, 200  $\mu$ M deoxyguanosine triphosphate and 0.5 U platinum Taq polymerase in the King Hot Start Taq polymerase reaction system (Ruian Biotech, China). The reaction involved an initial pre-denaturation for 3 min at 94°C, followed by 40 cycles with denaturation for 15 sec at 94°C, annealing and extension for 60 sec at 60°C in an ABI 7500 Fast Real-Time instrument. The final results are presented as methylated gene copies (*HOXD10*/*ALU*\*100).

The specific primers and TaqMan probes for the target gene *HOXD10* and the internal reference gene *ALU* are presented in Table II. The *HOXD10* gene and the promoter CpG island were searched for using the UCSC Human Genome Browser and PubMed (Fig. 1). The primers and TaqMan probes of *HOXD10* for Q-MSP assay were designed by JIELI Biotechnology (Shanghai, China). The specific primers and

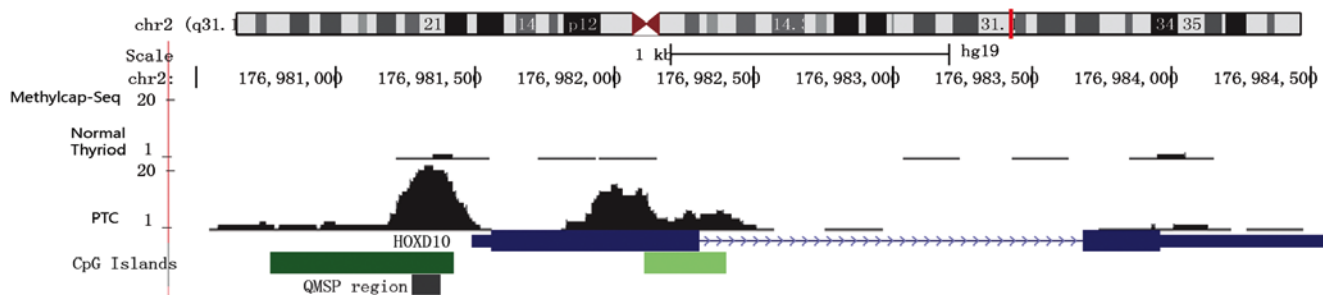


Figure 1. The chromosome ideogram and Q-MSP region of the *HOXD10* gene. The blue bar represents the *HOXD10* gene and the green bar represents the CpG island. The spectrum represents the methylation level in PTC and normal thyroid measured by MethylCap-seq. The black bar represents the fragment for Q-MSP.

TaqMan probes for the *ALU* gene were previously described by Weisenberger *et al* (18).

***BRAF* mutational screening.** *BRAF* gene mutational status was analyzed in both clinical samples and PTC cell lines. *BRAF* mutational screening was analyzed by PCR followed by DNA sequencing at Boshang Biotechnology (Shanghai, China). The specific PCR primers for the *BRAF*<sup>V600E</sup> mutation region are presented in Table II.

***RNA extraction and quantitative real-time PCR.*** Fresh-frozen tissue specimens were homogenized using a bead homogenizer. Genomic RNA from cell lines and tissues was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol.

The expression of the *HOXD10* gene was analyzed in both clinical samples and PTC cell lines by RT-PCR. Reverse transcription reaction was performed using 1 µg of total RNA with PrimeScript RT reagent kit with gDNA Eraser (Perfect Real-Time; RR047A; Takara, Dalian, Japan). The expression level of *HOXD10* was determined by RT-PCR using SYBR Premix Ex Taq (Tli RNaseH Plus; RR420A; Takara). The PCR reaction was performed in a 20-µl volume containing up to 100 ng of template cDNA in a Cyclelight 480 PCR system. The reaction involved an initial denaturation for 3 min at 94°C, followed by 40 cycles with denaturation for 5 sec at 94°C, annealing for 60 sec at 60°C. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The expression level of *HOXD10* was calculated using the 2<sup>-ΔCt</sup> method. The primers for RT-PCR were designed using the website [http://www.embnet.sk/cgi-bin/primer3\\_www.cgi](http://www.embnet.sk/cgi-bin/primer3_www.cgi). The RT-PCR primer sequences are shown in Table II.

***Statistical analysis.*** Statistical analyses were performed by Student's and paired t-tests, and Chi-square test. The odds ratios (ORs) for relationships between each variable and *HOXD10* methylation were calculated by univariate logistic regression analysis. Multivariate logistic regression analysis was used to analyze the relationship between invasion and other clinicopathological characteristics including the methylation status of *HOXD10*. All confidence intervals (CIs) were stated at the 95% confidence level. A value of P<0.05 was considered to be statistically significant. SPSS 19.0 was used for data analysis (SPSS, Inc., Chicago, IL, USA). Figures were constructed using GraphPad Prism 5, Adobe Illustrator CS4 and Stata/SE 12.0.

## Results

***HOXD10 promoter is hypermethylated in PTC tissues.*** Q-MSP was designed to detect the methylation level of *HOXD10* in PTC tissues. PTC and adjacent normal thyroid tissues (152 pairs) were tested by Q-MSP assay. The results of Q-MSP are shown in Fig. 2A. The overall methylation levels of the *HOXD10* promoter were significantly higher in PTC tissues than levels in the adjacent normal thyroid tissues (P=0.02). Our findings showed that the promoter region of the *HOXD10* gene was hypermethylated in 17.76% (27/152) of PTC tissues and in 10.53% (16/152) of adjacent normal thyroid tissues, using a cut-off value of 88 (*HOXD10*/*ALU*\*100) (Fig. 2B).

***HOXD10 mRNA expression is decreased in PTC tissues.*** To determine the relationship between the methylation status of the *HOXD10* gene and its expression level, we evaluated the *HOXD10* mRNA levels in 152 pairs of PTC tissue samples by RT-PCR. The result showed that the expression level of the *HOXD10* gene was significantly decreased in the PTC tissues when compared with the level in the adjacent normal thyroid tissues (P=0.008) (Fig. 3A). Low expression of *HOXD10* was found in 46.7% (71/152) of the PTC tissues and in only 13.8% (21/152) of the adjacent normal thyroid tissues (Table V), using a cut-off value of 0.06 (relative value).

***Hypermethylation and low expression of HOXD10 is associated with BRAF<sup>V600E</sup> mutation in PTC tissues.*** To analyze the relationship between DNA methylation and *BRAF* mutation, we tested the *BRAF*<sup>V600E</sup> mutation in 4 PTC cell lines (TPC-1, BCPAP, K1 and W3) and 152 PTC clinical samples. Our results showed that *BRAF*<sup>V600E</sup> mutation occurred in 64 (42.1%) PTC patients and in 2 PTC cell lines (BCPAP and K1). In accordance with previous studies, no *BRAF* mutation was found in TPC-1 and W3 cell lines. Moreover, we observed that the hypermethylation and low-expression of *HOXD10* was related to *BRAF*<sup>V600E</sup> mutation in PTC tissues. The expression of *HOXD10* was significantly lower in PTC tissues with *BRAF*<sup>V600E</sup> mutation than in those without the mutation (P=0.022) (Table V). However, the methylation status of *HOXD10* did not show statistical difference between PTC tissues with and without *BRAF*<sup>V600E</sup> mutation (P=0.669) (Table III). However, further analysis in PTC tissues with *BRAF*<sup>V600E</sup> mutation showed that the methylation levels of *HOXD10* were significantly higher in tumor tissues than levels in the adjacent normal thyroid tissues

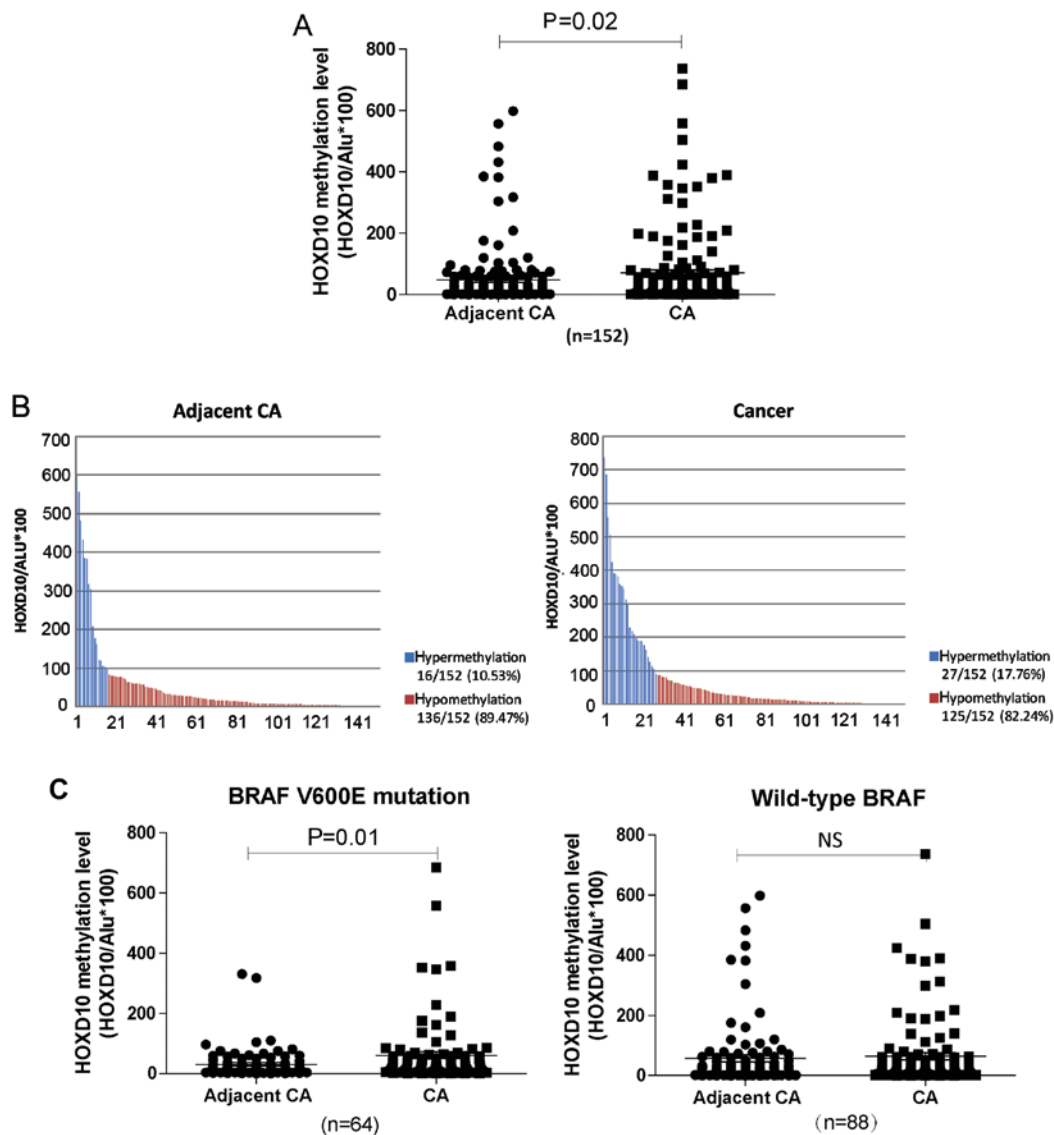


Figure 2. (A) Overall methylation levels of *HOXD10* in clinical samples by Q-MSP. The relative methylation level (on y-axis) is represented by ratios of *HOXD10*/ALU\*100. The error bars indicate the mean with 95% CI. The overall methylation levels of the *HOXD10* promoter were significantly higher in PTC tissues than in adjacent normal thyroid tissues ( $P=0.020$ ). (B) Hypermethylation grouping in PTC and adjacent normal thyroid tissues. The cut off value was determined by ROC curve, in which the methylation levels in PTC and adjacent normal tissues were used for analyses. The cut off value was 88. The relative methylation level (on y-axis) is represented by ratios of *HOXD10*/ALU\*100. (C) Methylation levels of *HOXD10* in *BRAF*<sup>V600E</sup> mutated PTCs and *BRAF* wild-type PTCs. The error bars indicate the mean with 95% CI. The methylation levels of *HOXD10* were significantly higher in tumor tissues than in adjacent normal thyroid tissues in *BRAF*<sup>V600E</sup> mutated PTCs ( $P=0.01$ ), while no significant difference was shown in *BRAF* wild-type PTCs ( $P=0.50$ ).

( $P=0.01$ ). While in PTC tissues without *BRAF*<sup>V600E</sup> mutation such a significant difference was not found ( $P=0.50$ ) (Fig. 2C).

**5-Aza-2-deoxycytidine treatment reverts the expression of the *HOXD10* gene in PTC cell lines.** To further verify the relationship between the methylation status of the *HOXD10* gene and its expression level, we detected the expression level of *HOXD10* and performed 5-Aza treatment in 4 PTC cell lines: TPC-1, W3 (*BRAF* wild-type) and BCPAP, K1 (*BRAF*<sup>V600E</sup> mutation) to ascertain whether the changes in the methylation level influence the expression of *HOXD10*. The results are shown in Fig. 3B. *HOXD10* mRNA was found to be weakly expressed in the TPC-1 and W3 cell lines, while the expression of *HOXD10* in the BCPAP and K1 cell lines showed no significant decrease compared with the normal thyroid tissues. After a 72 h treatment of 5-Aza, the expression of *HOXD10* in

the TPC-1 and W3 cell lines was significantly increased (57 and 396 times, respectively), while there were no significant changes in the expression of *HOXD10* in the BCPAP and K1 cell lines.

***HOXD10* suppresses the migration and induces the apoptosis of PTC cells.** To understand the potential functions of *HOXD10* in PTC, we overexpressed *HOXD10* in the TPC-1 cell line. TPC-1 cells were transfected with pcDNA3.1-*HOXD10* or pcDNA3.1 vector. The transfection efficiency was confirmed by RT-PCR and western blotting (Fig. 4A). We observed that the overexpression of *HOXD10* suppressed TPC-1 cell migration significantly compared to the control vector transfectants through a Transwell assay ( $P<0.01$ ; Fig. 4B). To explore the mechanisms underlying the inhibition of cell proliferation by the overexpression of *HOXD10*, we assessed cell apoptosis and

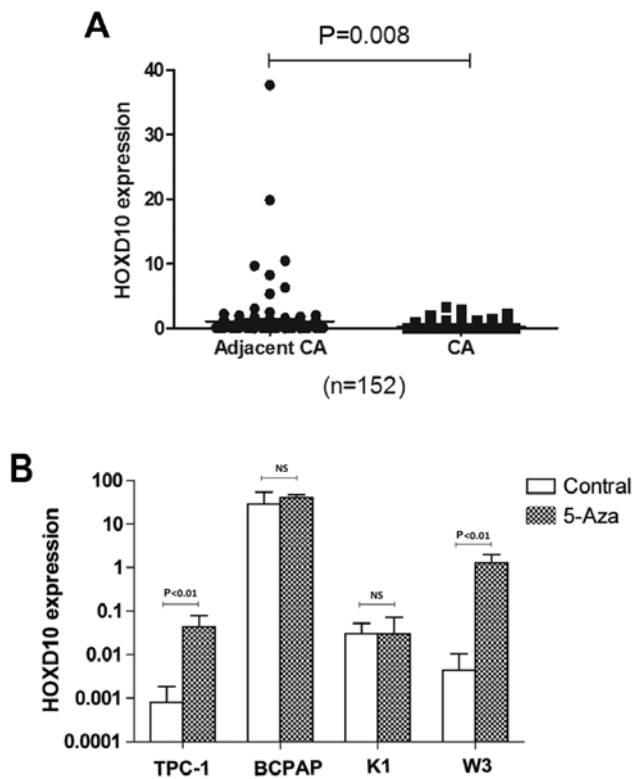


Figure 3. (A) Comparative expression profiling of *HOXD10* in clinical samples by RT-PCR. The expression level of *HOXD10* was calculated using the  $2^{-\Delta C_t}$  relative quantitation method. The average expression of adjusted normal thyroid tissue was relatively treated as 1. The error bars indicate the mean with 95% CI. The expression of *HOXD10* was significantly decreased in tumor tissues than that in adjusted normal thyroid tissue ( $P=0.008$ ). (B) Changes in expression levels of *HOXD10* in PTC cell lines after 5-Aza-2-deoxycytidine (5-Aza) treatment. The relative expression level (on y-axis) is represented by logarithmic form of  $\log_{10}$ . The average expression of normal thyroid tissue was relatively treated as 1. The error bars indicate the mean with 95% CI. The expression of *HOXD10* in TPC-1 and W3 cell lines was significantly increased after a 72-h treatment with 5-Aza, while there was no significant change in expression of *HOXD10* in the BCPAP and K1 cell lines.

cell cycle by flow cytometry. The overexpression of *HOXD10* induced the apoptosis of TPC-1 cells when compared to the empty vector-transfected cells. Additionally, we observed that the *HOXD10*-overexpressing TPC-1 cells showed higher S and G2 phase populations in comparison to the empty vector transfectants (Fig. 4C). In summary, the present study showed that the overexpression of *HOXD10* inhibited the migration of TPC-1 cells and also induced the apoptosis *in vitro*. The results implied that *HOXD10* may act as a tumor-suppressor in PTC.

*The aberrant hypermethylation of the HOXD10 gene is associated with clinicopathological characteristics.* The relationship between the *HOXD10* methylation status and clinicopathological characteristics was analyzed to evaluate the prognostic value of the *HOXD10* gene as a biomarker of PTC. Chi-square analysis and univariate logistic regression analysis revealed that age  $>45$  (OR 3.881, 95% CI 1.930-9.847;  $P=0.003$ ) and invasion (OR 2.972, 95% CI 1.157-7.633;  $P=0.027$ ) were associated with the hypermethylation status of *HOXD10* (Table III and Fig. 5), while, no relationship was found between *HOXD10* hypermethylation status and sex,

Table III. Association between clinicopathological factors and *HOXD10* methylation.

Characteristics	Hyper-methylated n (%)	Hypo-methylated n (%)	P-value
Patients	27 (17.8)	125 (82.2)	
Age (years)			<b>0.003<sup>a</sup></b>
>45	20 (27.4)	53 (72.6)	
≤45	7 (8.9)	72 (91.1)	
Sex			1.000
Male	6 (18.2)	27 (81.8)	
Female	21 (17.6)	98 (82.4)	
Invasion			<b>0.027<sup>a</sup></b>
Yes	9 (33.3)	18 (66.7)	
No	18 (14.4)	107 (85.6)	
Size (cm)			0.170
>1	16 (15.0)	91 (85.0)	
≤1	11 (24.4)	34 (75.6)	
Multifocality			1.000
Yes	8 (17.0)	39 (83.0)	
No	19 (18.1)	86 (81.9)	
Bilaterality			0.306
Yes	5 (16.7)	25 (83.3)	
No	22 (18.0)	100 (82.0)	
Hashimoto's thyroiditis			0.776
Yes	5 (20.0)	20 (80.0)	
No	22 (17.3)	105 (82.7)	
Lymph node metastasis			0.523
Yes	13 (15.5)	71 (84.5)	
No	14 (20.6)	54 (79.4)	
Central lymph node metastasis			0.405
Yes	12 (17.8)	67 (84.8)	
No	15 (20.5)	58 (79.5)	
Lateral lymph node metastasis			0.636
Yes	6 (14.3)	36 (85.7)	
No	21 (19.1)	89 (80.9)	
BRAF mutation			0.669
Yes	10 (15.6)	54 (84.4)	
No	17 (19.3)	71 (80.7)	

Data are presented as n (%). <sup>a</sup>Statistically significant ( $P<0.05$ ).

tumor size, multifocality, bilaterality, lymph node metastasis or Hashimoto's thyroiditis. The multivariate regression analysis was also performed to find the correlationship between invasion and other clinicopathological characteristics including methylation status of *HOXD10*. The result indicated that the hypermethylation of *HOXD10* (OR 3.779, 95% CI 1.283-11.128;  $P=0.016$ ) as well as tumor size  $>1$  cm (OR 7.456,

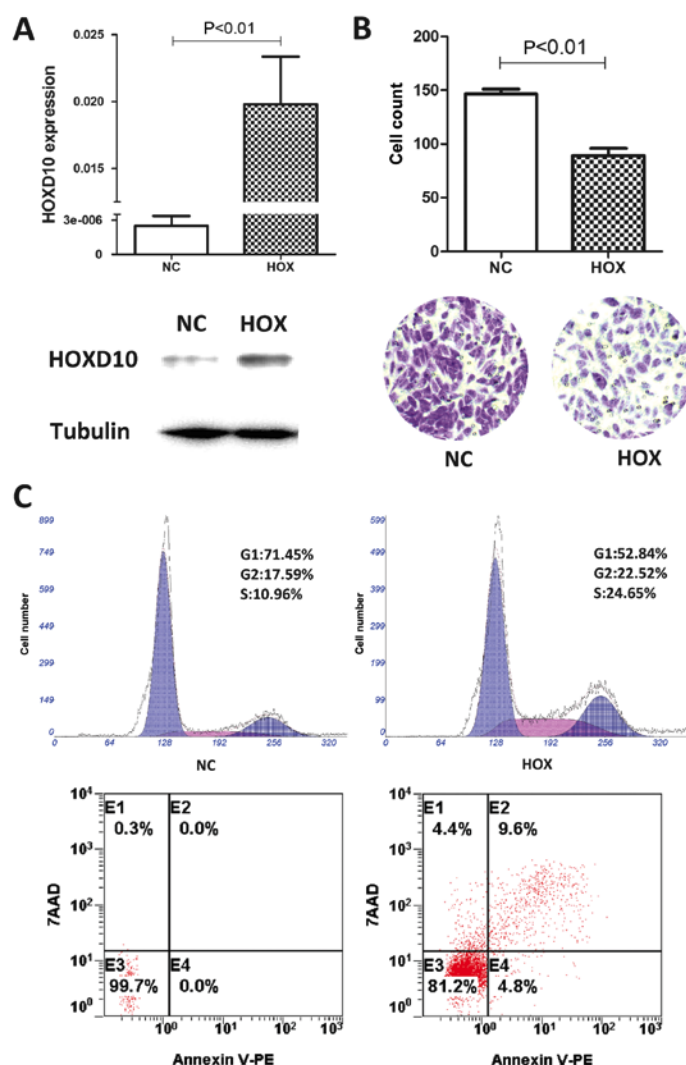


Figure 4. (A) The confirmation of cell transfection efficiency by RT-PCR and western blotting.  $\beta$ -actin and tubulin were used as endogenous control genes. RT-PCR values are expressed as relative expression with respect to the endogenous control gene,  $\beta$ -actin. The RNA and protein expression of *HOXD10* were significantly increased after transfection with pcDNA3.1-*HOXD10* compared with the empty vector-transfected TPC-1 cells. (B) Cell migration assays were performed in modified Boyden Transwell chambers assay. TPC-1 cells/well ( $2 \times 10^4$ ) were plated and incubated for 12 h. The migratory cells on the bottom of the membrane were fixed and stained with polyfluoroalkoxy (PFA) and crystal violet stain solution (0.5%). The mean number of visible cells was counted by fluorescence microscope in 5 random high power fields. (C) Cell cycle distribution and the rate of cell apoptosis after transfection of the TPC-1 cells with *HOXD10* was analyzed by flow cytometry. The mean percentages of cell cycle phases from representative experiments are shown in each panel. Region E1 indicates early apoptotic cells; E2 shows late apoptotic cells.

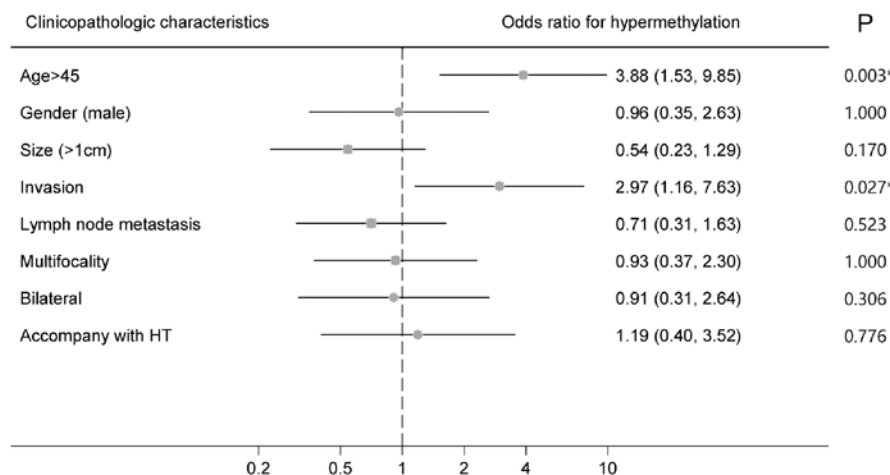


Figure 5. Univariate logistic regression analysis of clinicopathological characteristics and *HOXD10* methylation. The bars represent OR and 95% CI. Age >45 (OR 3.881, 95% CI 1.930-9.847;  $P=0.003$ ) and invasion (OR 2.972, 95% CI 1.157-7.633;  $P=0.027$ ) were associated with the hypermethylation status of the *HOXD10* promoter. \*Statistically significant.

Table IV. Multivariate regression analysis of invasion in PTC.

Characteristics	OR (95% CI)	P-value
Sex (male)	1.035 (0.341-3.141)	0.951
Age (years)	2.044 (0.773-5.408)	0.150
Size >1 cm	7.456 (1.484-37.459)	<b>0.015<sup>a</sup></b>
Lymph node metastasis	1.579 (0.556-4.483)	0.391
Multivariate	0.262 (0.065-1.054)	0.059
Bilateral	2.432 (0.565-10.474)	0.233
Hashimoto's thyroiditis	0.593 (0.119-2.968)	0.525
HOXD10 hypermethylation	3.779 (1.283-11.128)	<b>0.016<sup>a</sup></b>

Data are presented as OR (95% CI). <sup>a</sup>Statistically significant (P<0.05). PTC, papillary thyroid cancer; CI, confidence interval.

95% CI 1.484-37.459; P=0.015) were independent risk factors of invasion in PTC (Table IV). In a word, our results showed the potential clinical value of *HOXD10* methylation in PTC as a biomarker.

The relationship between *HOXD10* expression and other clinicopathological characteristics including *BRAF*<sup>V600E</sup> mutation was also analyzed. Age >45 (P=0.022) and *BRAF*<sup>V600E</sup> mutation (P=0.022) were found to be associated with low expression of *HOXD10* (Table V). However, no relationship was found between *HOXD10* expression status and sex, tumor size, invasion, multifocality, bilaterality, lymph node metastasis or Hashimoto's thyroiditis. In addition, no significant correlation was found between *BRAF*<sup>V600E</sup> mutation and other clinicopathological characteristics.

## Discussion

DNA methylation is one of the most common molecular events in cancers, along with genetic alterations, leading to carcinogenesis. Evaluating the status of DNA methylation could be useful for the diagnosis and prognostic evaluation of cancers and may be helpful in clarifying the process of tumorigenesis (12,19-22). We have paid close attention to DNA methylation in thyroid cancer in recent years. Our previous studies established a genome-wide DNA methylome database of PTC by MethylCap-seq, demonstrating that the *HOXD10* gene was aberrantly hypermethylated in PTC.

Previous studies recognized *HOXD10* as a sequence-specific transcription factor, mainly involved in cell differentiation and limb development (23,24). In recent years, its function in tumorigenesis has been gradually recognized (25,26). The homeobox superfamily plays an important role in cell differentiation and morphogenesis. The dysregulation of the *HOX* gene may affect various pathways and play roles in tumorigenesis and metastasis (25,27). Several *HOX* genes (such as *HOXB13*, *HOXA5* and *HOXC6*) have been found aberrantly expressed through promoter methylation in malignancies including lung, breast and gastrointestinal cancer (25,26,28-33). Emerging

Table V. Association between clinicopathological factors of the PTC cases and *HOXD10* expression.

Characteristics	High expression n (%)	Low expression n (%)	P-value
Patients	81 (53.3)	71 (46.7)	
Age (years)			<b>0.022<sup>a</sup></b>
>45	37 (44.6)	46 (55.4)	
≤45	44 (63.8)	25 (36.2)	
Sex			0.333
Male	15 (45.5)	18 (54.5)	
Female	66 (55.5)	53 (44.5)	
Invasion			0.839
Yes	16 (55.2)	13 (44.8)	
No	65 (52.8)	58 (47.2)	
Size (cm)			0.237
>1	47 (50.0)	47 (50.0)	
≤1	34 (60.7)	22 (39.3)	
Multifocality			0.490
Yes	29 (58.0)	21 (42.0)	
No	52 (51.0)	50 (49.0)	
Bilaterality			0.839
Yes	15 (50.0)	15 (50.0)	
No	65 (53.7)	56 (46.3)	
Hashimoto's thyroiditis			0.829
Yes	14 (56.0)	11 (44.0)	
No	67 (52.8)	60 (47.2)	
Lymph nodes metastasis			0.254
Yes	41 (48.8)	43 (51.2)	
No	40 (58.8)	28 (41.2)	
Central lymph node metastasis			0.197
Yes	38 (38.1)	41 (51.9)	
No	43 (58.9)	30 (41.1)	
Lateral lymph node metastasis			0.857
Yes	23 (54.8)	19 (45.2)	
No	58 (52.7)	52 (47.3)	
BRAF mutation			<b>0.022<sup>a</sup></b>
Yes	27 (42.2)	23 (57.8)	
No	54 (61.4)	34 (38.6)	

Data are presented as n (%). <sup>a</sup>Statistically significant (P<0.05).

studies have found that the expression of *HOXD10* is decreased in various tumors (such as breast and gastric cancer), and have considered *HOXD10* as a candidate tumor-suppressor gene (34-39). However, the methylation and expression status of the *HOXD10* gene, and its biological significance in PTC have not been identified.

In the present study, 152 pairs of PTC samples were collected for relative research, including Q-MSP, RT-PCR and *BRAF* mutation sequencing. Cytology experiments with 4 PTC cell lines were carried out to explore the relationship between the methylation and expression status of *HOXD10*. Overexpression transfection of *HOXD10* in TPC-1 cells was designed to research the function of *HOXD10* in PTC. The results showed that the methylation level of the *HOXD10* gene was significantly higher in PTC tissues, compared with that noted in the adjacent normal thyroid tissues ( $P=0.02$ ). RT-PCR assay showed that the expression of *HOXD10* was significantly decreased in PTC cell lines and tumor tissues than that observed in the adjacent normal thyroid tissues ( $P=0.008$ ), which was in accordance with the Q-MSP results. 5-Aza treatment reverted the expression of the *HOXD10* gene in PTC cell lines, which demonstrated that the decreased expression of *HOXD10* was caused by aberrant promoter hypermethylation. Moreover, the overexpression of *HOXD10* suppressed the migration of TPC-1 cells, and promoted the cell apoptosis, implying that *HOXD10* may act as a tumor suppressor in PTC. In addition, statistical analysis showed the potential clinical value of *HOXD10* methylation as a biomarker. Besides, further stratified analysis showed that low expression of *HOXD10* was related to *BRAF*<sup>V600E</sup> mutation ( $P=0.022$ ). Also, the *HOXD10* methylation level of PTC was significantly higher than that of adjacent normal thyroid tissues in patients with *BRAF*<sup>V600E</sup> mutation ( $P=0.01$ ). In conclusion, we found that the *HOXD10* gene was downregulated through promoter hypermethylation in PTC and *HOXD10* may act as a tumor suppressor. Moreover, the aberrant hypermethylation and low expression of *HOXD10* were associated with *BRAF*<sup>V600E</sup> mutation in PTC.

It is widely accepted that PTC with *BRAF*<sup>V600E</sup> mutation is a flag of high-risk. Detection of *BRAF*<sup>V600E</sup> mutation is an important clinical application for the diagnosis and prognostic prediction of PTC. In addition, the present study suggests that *HOXD10* may be a candidate tumor suppressor and it also has interactions with the *BRAF* gene. The results showed that the low expression of *HOXD10* was associated with *BRAF*<sup>V600E</sup> mutation ( $P=0.022$ ). In addition, in PTCs with *BRAF*<sup>V600E</sup> mutation, the methylation levels of *HOXD10* were significantly higher in PTC tissues than in adjacent normal thyroid tissues. While no significant difference was observed in PTCs without *BRAF*<sup>V600E</sup> mutation. In addition, the PTC cell lines with *BRAF*<sup>V600E</sup> mutation (K1, BCPAP) showed relatively high expression of *HOXD10* and low sensitivity to 5-Aza treatment. On the contrary, the PTC cell lines without *BRAF*<sup>V600E</sup> mutation (TPC-1, W3) showed relatively low expression of *HOXD10* and high sensitivity to 5-Aza treatment. The results indicated the possible interaction between *HOXD10* gene and *BRAF*<sup>V600E</sup> mutation. One possible hypothesis to explain the results was that the hypermethylation of *HOXD10* may be an accompanied event in *BRAF*<sup>V600E</sup>-mutated PTC, where *BRAF*<sup>V600E</sup> mutation plays the major role in tumorigenesis. While in wild-type PTC, *HOXD10* hypermethylation may play an important role in tumorigenesis. Combining the detections of *HOXD10* methylation and *BRAF* mutation may be a good choice to improve clinical diagnostic and prognostic accuracy.

Our results showed that the *HOXD10* gene may be involved in PTC tumorigenesis and it may act with *BRAF*<sup>V600E</sup> mutation. However, the underlying mechanism of *HOXD10* is still not

clear. Wang *et al* (39) reported that *HOXD10* regulates multiple downstream genes including *IGFBP3* in gastric cancer. Reintroduction of *HOXD10* upregulated *IGFBP3*, activated caspase-3 and caspase-8, and subsequently induced cell apoptosis. Yang *et al* (40) claimed that *HOXD10* acted as a tumor suppressor via the inhibition of RHOC/AKT/MAPK pathway in cholangiocellular carcinoma. The upregulation of *HOXD10* led to the dephosphorylation of AKT and ERK, implying that the PI3K/AKT and MAPK pathways were significantly inactivated. According to the above studies, MAPK pathways may be a key point for the interaction between *BRAF* and *HOXD10*, since *BRAF* is one of the most important regulatory gene in the MAPK pathways. However, further research is needed to clarify the mechanisms of the interaction between *BRAF* and the *HOXD10* gene.

Combined with clinical data, the hypermethylation status of the *HOXD10* promoter was significantly correlated with age >45 (OR 3.881, 95% CI 1.930-9.847;  $P=0.003$ ) and invasion (OR 2.972, 95% CI 1.157-7.633;  $P=0.027$ ). These 2 clinical characteristics usually predict a worse prognosis of PTC. Patients with an age >45 years and patients with primary tumor invasion may have a higher chance of recurrence and metastasis, leading to worse survive (41-43). Our analysis indicated that the hypermethylation of the *HOXD10* gene was an independent risk factor for invasion in PTC. This indicates that the *HOXD10* gene may play a role in PTC tumorigenesis and its methylation status could be used to predict the prognosis of PTC as a biomarker.

Recently, the incidence of thyroid cancer particularly PTC has significantly increased (2,44). It is well known that patients with PTC usually have good prognosis, but a small population of patients have a relatively higher recurrence risk (5,42). New biomarkers are urgently needed for the diagnosis and prognostic prediction of PTC. Currently, several biomarkers have been used to improve the diagnostic accuracy in PTC, such as the detection of *BRAF*<sup>V600E</sup> mutation (45-49). DNA methylation of tumor suppressors (such as *Rassf1A* and *RARβ2*) and thyroid-specific genes (such as *TSHR*) have been determined to be associated with *BRAF*<sup>V600E</sup> mutation, which may also play a role in PTC tumorigenesis (50-52). However, apart from genetic biomarkers, research must provided new ideas to search for viable epigenetic biomarkers to improve the diagnostic and prognostic accuracy in PTC. According to the present study, the hypermethylation of *HOXD10* may be a promising biomarker for the diagnosis and prognostic prediction of PTC.

In summary, the present study firstly studied the methylation profile of *HOXD10* and explored its functions in PTC. *HOXD10* may act as a tumor suppressor in PTC. The decreased expression of the *HOXD10* gene caused by aberrant hypermethylation was shown in PTCs particularly in those with *BRAF*<sup>V600E</sup> mutation. The epigenetic suppression of the *HOXD10* gene may play a role in the tumorigenesis of PTC, and it may be a prospective biomarker for the diagnosis and prognostic prediction of PTC.

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