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Original Article

# Methylation of p16 CpG islands correlated with metastasis and aggressiveness in papillary thyroid carcinoma

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**Abstract**

*Background*: Inactivation of p16 by methylation of CpG islands is a frequent early event in human cancers, including papillary thyroid carcinoma (PTC). This study was to observe the methylation status of the p16 gene in papillary thyroid carcinoma (PTC) and its correlation with clinical parameters.
*Methods*: Methylation-specific PCR (MSP) was used to analyze the methylation status of the p16 gene in 74 PTCs and 21 adjacent normal thyroid tissues.
*Results*: Hypermethylation of p16 gene was observed in 27.0% (20/74) of PTC. None of the normal thyroid tissues was methylated, when compared to the PTCs ($p < 0.05$). There was no marked relationship between the methylation of p16 gene and the patients' age, gender, size of cancer, histological subtypes and occurrence of recurrent disease ($p > 0.05$). The methylation of p16 gene was positively associated with metastasis, a high AMES (age, metastasis to distant sites, extrathyroidal invasion, size) risk group ($p < 0.05$) and advanced pathological tumor−lymph node−metastasis stages.
*Conclusion*: The methylation of the p16 gene, one event of significance in molecular biology, was common and correlated with biological metastasis and histological features in PTC, and may be involved in thyroid tumorigenesis and aggressiveness.


*Keywords:* CpG islands; methylation; P16; papillary thyroid carcinoma

## 1. Introduction

The P16 suppressor gene is one of the most commonly studied candidates in the pathogenesis of human neoplasia, belonging to a class of cyclin-dependent kinase (CDK) inhibitory proteins. It binds specifically to CDK 4 and inhibits the catalytic activity of the CDK-cyclin D complex, a key regulator of the G1 checkpoint of the cell cycle.[1] There are three main mechanisms that alter the p16 gene: homozygous deletion, promoter hypermethylation, and, very rarely, point mutations.[2] In particular, promoter hypermethylation of CpG islands has been shown to be an epigenetic change resulting in loss of function of responsive genes involved in cell cycle regulation and DNA repair. Methylation of p16 CpG islands silences transcription of this gene.[3] It has been observed that in primary gastric carcinoma (GC), the frequency of p16 inactivation by homozygous deletions ranges 0−9%, by mutation 0−2%, while by methylation 32−42%,[4−10] which suggests that methylation is a major mechanism for p16 inactivation in GC. Moreover, p16 methylation is significantly correlated with progression of GCs.[11] In addition, it was reported that the p16-

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methylated cells could have an advantage in progression and metastasis in non-small-cell lung cancers.[12]

Papillary thyroid carcinoma (PTC) is the most common malignancy originating in the thyroid gland, and accounts for about 60% of adult thyroid carcinomas and 100% of childhood thyroid carcinomas. With respect to gene alterations in carcinogenesis, RET, BRAF, and RASSF1A genes have been noted in a substantial number of patients with PTC.[13,14] However, our understanding of the pathway toward carcinogenesis in PTC is incomplete, in part because abnormalities in the control of cell cycle checkpoints are common in carcinogenesis. As for p16 inactivation in PTC, some previous studies[15−18] have demonstrated that hypermethylation of the 5′ CpG island of p16 are common and critical events, responsible for the development of PTC, rarely by mutation. Moreover, Lam et al.[18] reported p16 mRNA or p16 protein was detected in 77% and 89% of PTC, respectively, indicating that the overexpression of p16 rather than the loss of its protein contributes to the pathogenetic mechanism of PTC. These findings imply that methylation of CpG islands of p16 may also play a role in increasing the occurrence and metastatic potential of PTC as an epigenetic event during disease progression.

In this study, a total of 74 PTC tissues and 21 adjacent normal thyroid tissues were evaluated using methylation-specific PCR (MSP) to assess hypermethylation in the p16 gene implicated in regulatory processes, revealing a progressive increase of p16 alterations within thyroid tumorigenesis and from primary carcinomas to metastatic lesions. The results showed statistically significant correlations between p16 methylation and certain clinical features of the tumors.

## 2. Methods

### 2.1. PTC samples and cell lines

A total of 74 PTCs including 37 primary and 37 metastatic tumors, and 21 adjacent normal thyroid tissues from primary (10 cases) and metastatic (11 cases) tumors were obtained from patients from the Beijing Cancer Hospital by surgical resection (with their informed consent) between 2004 and 2010 and were stored at −70°C until use. Treated patients received routine clinical and sonographic follow-up every 3−6 months. The follow-up period ranged from 6 months to 7 years 9 months (mean, 21 months). During the follow-up period, cervical lymph node involvement was verified in five patients (6.76%). Distant metastases (bone or lung) developed in two patients (2.63%). All clinical samples and histopathological information for each case were obtained according to approved institutional guidelines. A tumor risk profile was assigned according to commonly adopted AMES (age, metastasis to distant sites, extrathyroidal invasion, size) risk group stratification and the Union for International Cancer Control (UICC) pTNM (pathological tumor−lymph node−metastasis) staging.

Human gastric cancer cell line AGS and MGC803, serving as p16 promoter methylation and unmethylation, were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) at 37°C with 5% $CO_2$.

This study was approved by the Research and Ethical Committee of Peking University School of Oncology.

### 2.2. DNA extraction

DNA was extracted from frozen thyroid tissue and tissue culture cells by standard sodium dodecyl sulfate (LookChem, Shijiazhuang, China)/proteinase K (SBS Genetech, Beijing, China) digestion followed by organic extraction and ethanol precipitation.

### 2.3. Sodium bisulfite treatment

One microgram of genomic DNA was denatured in 0.2 M NaOH (Guide Chem, Beijing, China) for 10 minutes at 37°C. The denatured DNA was diluted in 500 μL of freshly prepared solution of 10 mM hydroquinone (Yaou Chemicals Co. Ltd, Taiyuan, China) and 3 M sodium bisulfite (JinRuiQi Chemical Co. Ltd, Tianjin, China) and incubated for 16 h at 50°C. After incubation, the DNA sample was desalinized through a column (Wizard DNA Clean-Up System; Promega, Madison, WI, USA), treated with 0.3 M NaOH for 10 minutes at room temperature, and precipitated with ethanol. The bisulfite-modified genomic DNA was resuspended in 40 μL of $H_2O$ and stored at −20°C.

### 2.4. Methylation-specific PCR of p16

DNA methylation status for all cell lines and PTC were determined by MSP, which is sufficiently sensitive to detect a single methylated allele among 1000 unmethylated ones and, therefore, may be the most appropriate methodology for identifying DNA methylation patterns within CpG islands of p16. MSP distinguishes unmethylated from methylated alleles based on sequence changes produced after bisulfite treatment of DNA, which converts only unmethylated cytosine to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA.

Therefore, we utilized MSP to analyze the methylation status of the bisulfite-modified p16 CpG islands. Both the p16-M and p16-U primer sets for the methylated p16 CpG islands.[19] were 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (sense p16-M), 5′-GACCCCGAACCGCGACCGTAA-3′ (antisense p16-M), 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ (sense p16-U), and 5′-CAACCCCAAACCACAACCATAA-3′ (antisense p16-U).

The PCR products of p16 CpG islands were amplified by hot-start PCR. HotStarTaq DNA polymerase (QIAGEN GmbH, Hilden, Germany) was used. Thermal cycler conditions were denaturing at 95°C for 15 minutes, amplified for 35 cycles (95°C for 40 seconds, 62°C for 40 seconds, 72°C for 40 seconds), with extension at 72°C for 10 minutes. The reaction mixture (20 μL) contained about 10 ng of templates, 4 pmol of each primer, 4 nmol of dNTP, and 1 unit of HotStarTaq DNA polymerase. Distilled water and genomic DNA of the p16 unmethylated MGC803 cells were used as template