European Journal of Inflammation

*Letter to the Editor*

# Relationship between DAPK methylation and gene inactivation in papillary thyroid carcinoma

European Journal of Inflammation
Volume 16: 1–6

DOI: 10.1177/2058739218778710
journals.sagepub.com/home/eji
SAGE

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**Abstract**

To investigate the relationship between the methylation of death-associated protein kinase (DAPK) promoter and gene inactivation in papillary thyroid carcinoma, the technique of methylation-specific polymerase chain reaction (PCR (MSP)) was applied to detect the methylation status of DAPK gene promoter in 70 cases of papillary thyroid carcinoma (study group) and in 50 cases of corresponding adjacent tissues (control group). Immunohistochemical method was used to detect the protein expression; besides, the relationship of DAPK methylation and gene inactivation with pathological factors of papillary thyroid cancer was analyzed. The methylation rate of DAPK was 16% (8/50) in the control group and 71.4% (50/70) in the study group with the difference being statistically significant ($\chi^2 = 19.724$, $P < 0.01$). The methylation of DAPK gene promoter was not associated with age, sex, tumor size, TNM stage, and thyroid capsular infiltration in the study group with papillary thyroid carcinoma ($P > 0.05$), but was associated with lymph node metastasis ($P < 0.05$). Spearman's rank correlation analysis showed that the methylation of DAPK promoter was negatively correlated with the expression of DAPK ($r = -0.793$, $P < 0.01$). The methylation of CpG island in the promoter region of DAPK gene can lead to gene inactivation and may be involved in the occurrence of papillary thyroid carcinoma.

**Keywords**

DAPK, methylation, thyroid papillary carcinoma, tumor suppressor gene

Date received: 4 February 2018; accepted: 26 April 2018

## Introduction

Thyroid cancer is the most common malignant endocrine tumor and also one of the malignant tumors with the most rapid increase of incidence in recent years.[1] It has high incidence in coastal countries and regions with high rate of external invasion and common distant metastasis. Papillary thyroid carcinoma (PTC) ranks in the first place in thyroid cancer, accounting for about 75%.[2] In recent years, patients with PTC tend to be younger and younger. Methylation is one of the most common molecular alterations in cancer, including a decrease in total genome methylation and the occurrence of methylation in certain promoter regions.[3–5] Related studies have found that P16, P53, and Ras ras-associated domain family 1A gene and E cadherin are frequently methylated in human thyroid cancer.[6] Aberrant methylation of genes leads to abnormal transcription and thus plays an important role in the development and progression of thyroid cancer.[7] The effect of aberrant gene methylation is an important molecular biological mechanism for

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researches into the occurrence and development of thyroid cancer. A common feature of human tumors is inactivation of many tumor-suppressor genes or tumor-associated genes caused by the CpG island methylation, which is usually concerned with blocking and deletion of the related proteins.[8] Death-associated protein kinase (DAPK) is a 160 Ku calmodulin (CaM) regulated serine/threonine protein kinase and a positive regulator of apoptosis, which is closely related to the occurrence, development, and metastasis of tumors.[9] DAPK can be activated by a variety of factors such as INF-γ, Fas, TNF-β, and ERK, suggesting that it may be the convergence of various signals which induce apoptosis. The methylation of DAPK promoter is a hot spot in the field of research on tumor. This experiment adopts the technique of methylation-specific polymerase chain reaction (PCR(MSP)) to detect the CpG island methylation of DAPK promoter in thyroid papillary carcinoma and the adjacent normal tissues so as to study its relationship with the expression of DAPK and as well as the role of DAPK expression in the development of thyroid papillary carcinoma, expecting to further figure out the pathogenesis of PTC.

## Materials and methods

### *Materials*

Our study was approved by the Ethics committee of the affiliated hospital of Qingdao University. All patients provided written informed consent. A total of 70 patients with thyroid papillary carcinoma (study group) were selected from those treated by surgical resection in our hospital from February 2015 to June 2017, with all confirmed by histopathological examination. They were aged 24–3 with the mean age of 50 years; the tumor diameter was less than 2 cm in 37 cases and more than 2 cm in 33 cases; and they were classified as follows: TNM staging: 40 stage I cases, 20 stage II cases, 8 stage III cases, 2 stage IV cases; 30 cases with capsular infiltration, 40 cases without capsular infiltration; 42 cases with lymph node metastasis, and 28 cases without lymph node metastasis. All cases underwent initial treatment and were diagnosed with PTC by histopathological examination. Exclusion criteria: The patient was diagnosed as a secondary thyroid tumor or treated with radiotherapy or chemotherapy before the operation.

Another 50 cases of corresponding adjacent tissues (more than 2 cm away) were selected as the control group. Fresh samples were collected from them for extraction of total DNA followed by paraffin embedding for immunohistochemistry detecting. All specimens were confirmed by two pathologists.

### *Main reagents and instruments*

Genomic DNA extraction kit was purchased from Beijing Solarbio Technology Co. Ltd; hydroquinone, sodium bisulfite from Nanchang Mingrui Chemical Co. Ltd; Wizard Cleanup DNA purification recovery system from Promega company; 20 bp DNA Ladder Marker, Taq™ Hot Start Version from TaKaRa company; DAPK primer by Beijing Aoke peak Biotechnology Co. Ltd. DAPK Rabbit antihuman polyclonal antibody from Shanghai Xinyu Biological Technology Co. Ltd; streptavidin peroxidase (SP) kit from Beijing Aoweiya Biotechnology Co., Ltd.; DAB reagent from Wuhan Boster Biological Engineering Co. Ltd; centrifugal machine from Beijing Huake Century Experimental Instrument Co. Ltd.; Ultraviolet spectrophotometer from Shanghai Analytical Instrument Factory; PCR device from Beijing's Far East Science Instrument Co. Ltd.; electrophoresis device from Beijing Baijing Biotechnology Co. Ltd; and Gel imaging system from Beijing mystage Tech Co. ltd..

### *DNA extraction and bisulfite sodium modification*

The genomic DNA were extracted according to the kit instructions. The DNA sample (1–2 μg) was diluted to 50 μL with sterile water followed by additions of 5.5 μL freshly prepared NaOH (3 mol/L), water bath at 42°C for 30 min, 30 μL hydroquinone (10 mmol/L), 520 μL sodium bisulfite (3.6 mol/L), cover with mineral oil, water bath at 50°C for 16 h away from light, DNA purification by Wizard DNA purification and recovery system, ethanol precipitation, the addition of 20 μL sterile water, and the restoration at −20°C.

### *MSP detection*

Amplification of DAPK was performed by MSP method. DAPK methylation primer: 5-GGATAGT CGGATCGAGTTAACGTC-3' 3 (sense sequence), 5′-CCCTCCCAAAC-GCCGA-3′ (antisense sequence), amplified fragment 98 bp. Non-methylated primers: 5 '-GGAGGATAGTTG GATTGAGTTA

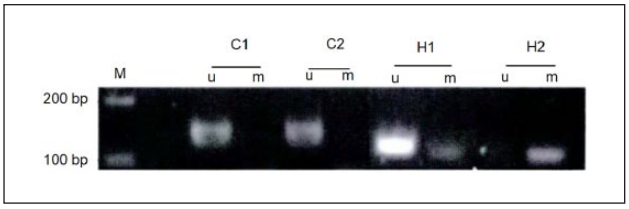


**Figure 1.** Electrophoresis results of DAPK methylation in the two groups.
M: Marker; u: without methylation in CG sites; m: with methylation in CG sites; C1 and C2: control group specimens; H1 and H2: study group specimens.

ATGTT-3' 3 (sense sequence), 5 '-CAAATCCC TCCCAAACACCAA-3' 3(antisense sequence), amplified fragment 106 bp. Reaction system (25 μL): 10 × PCR buffer 2 μL, 4 μL dNTP mixture (2.5 mmol/L), 0.3 μL Taq hot start (HS) enzyme, 1 μL primers, 2 μL template DNA, 14.7 μL $H_2O$. The reaction conditions were as follows: 95℃ 5 min, 94℃ 45 s, 60℃ 45 s, 72℃ 45 s, after 35 cycles, 70℃ 7 min. DNA of human peripheral blood lymphocytes and Sss I methyltransferase treated placental DNA and water were, respectively, used as positive control and blank control for non-methylation and methylation.

### *Immunochemistry*

The expression of DAPK was detected by immunochemistry (SP method) with the experimental procedures carried out according to the instruction manual. The DAPK primary antibody was applied overnight at 4 C at a dilution 1:200. Phosphate buffered saline (PBS) was used as blank control instead of the first antibody. Secondary antibodies were applied at a dilution 1:1000 for 2 h at room temperature. The known specimens with DAPK positive were sectioned for positive control and the known specimens with DAPK negative were sectioned for negative control. Positive expression suggested the cytoplasm was brownish yellow staining. Pictures were taken with an Olympus Fluorescence Microscope OlympusBX40 and an Olympus XM10 camera.

### *Statistical analysis*

Statistical analysis was carried out on SPSS 21 statistical software. The positive rate between groups was compared by $\chi^2$ test or Fisher's exact test, and the correlation test was performed by Spearman's rank analysis, with "$\alpha = 0.05$" as the standard of significant test.

**Table 1.** Methylation of DAPK gene promoter in the two groups of specimens.

| Study group | Control group | | Sum |
|---|---|---|---|
| | (−) | (+) | |
| (−) | 7 | 1 | 8 |
| (+) | 43 | 7 | 50 |
| Sum | 50 | 8 | 58 |

DAPK: death-associated protein kinase.

## Results

### *Methylation status of DAPK promoter*

The methylation rate of DAPK was 16% (8/50) in the control group and 71.4% (50/70) in the study group with the difference being statistically significant, as shown in Figure 1 and Table 1.

### *Relationship between DAPK methylation and clinicopathological factors in PTC*

The methylation of DAPK gene promoter was not associated with age, sex, tumor size, TNM stage, and thyroid capsular infiltration in the study group with PTC ($P > 0.05$) but was associated with lymph node metastasis ($P < 0.05$), as shown in Table 2.

### *Relationship between DAPK promoter methylation and DAPK expression*

In this study, there was a sum of 58 methylated thyroid specimens in both groups, of which 8 cases were positive DAPK expression and 50 cases were negative. In another 62 cases of non-methylated thyroid specimens, 56 cases were positive DAPK expression and 6 cases negative. Spearman's rank correlation analysis showed a negative correlation between methylation of DAPK promoter and expression of DAPK protein ($r = -0.793$, $P < 0.01$). As shown in Figures 2 and 3.

Summary: the methylation rate of DAPK was 16% (8/50) in the control group and 71.4% (50/70) in the study group with the difference being statistically significant ($\chi^2 = 19.724$, $P < 0.01$); the methylation of DAPK gene promoter was not associated with age, sex, tumor size, TNM stage, and thyroid capsular infiltration in the study group with PTC

**Table 2.** Relationship between DAPK methylation and clinicopathological factors in papillary thyroid carcinoma (case).

| Clinicopathological factors | n | DAPK methylation | | $X^2$ | *P* |
|---|---|---|---|---|---|
| | | (+) | (−) | | |
| Gender | | | | 1.308 | >0.05 |
| Male | 37 | 26 | 11 | | |
| Female | 33 | 24 | 9 | | |
| Age (year) | | | | 0.941 | >0.05 |
| ≤50 | 32 | 23 | 9 | | |
| >50 | 38 | 27 | 11 | | |
| Thyroid capsular infiltration | | | | 0.732 | >0.05 |
| With | 30 | 21 | 9 | | |
| Without | 40 | 29 | 11 | | |
| Tumor size(cm) | | | | 1.564 | >0.05 |
| ≤2 | 37 | 25 | 12 | | |
| >2 | 33 | 25 | 8 | | |
| TNM stage | | | | 1.761 | >0.05 |
| I+II | 60 | 52 | 8 | | |
| III+IV | 10 | 8 | 2 | | |
| Lymph node metastasis | | | | | <0.05 |
| With | 42 | 39 | 3 | 7.091 | |
| Without | 28 | 11 | 17 | | |

DAPK: death associated protein kinase.

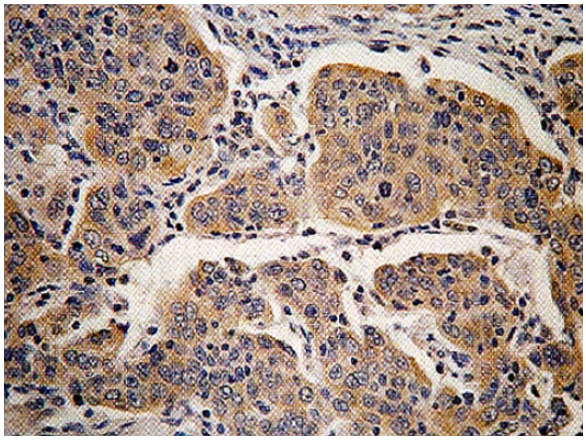

**Figure 2.** The positive expression of DAPK protein in methylated thyroid specimens (SP × 40).

($P>0.05$), but was associated with lymph node metastasis ($P<0.05$); and the Spearman's rank correlation analysis showed that the methylation of DAPK promoter was negatively correlated with the expression of DAPK ($r=-0.793$, $P<0.01$). These results suggest that the methylation of CpG island in the promoter region of DAPK gene can lead to gene inactivation and may be involved in the occurrence of PTC.

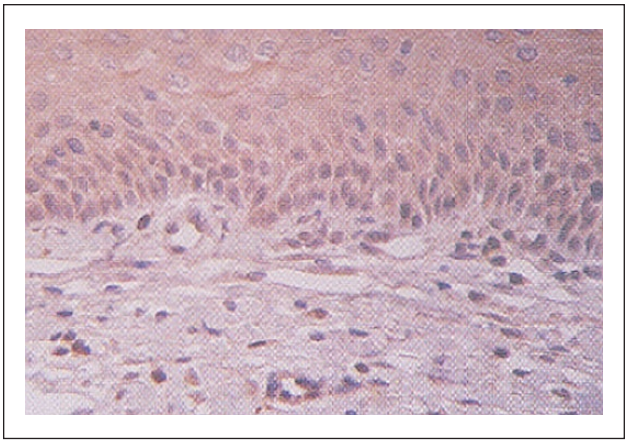

**Figure 3.** The positive expression of DAPK protein in non-methylated thyroid specimens (SP × 40).

## Discussion

Some researches on thyroid tumors suggest that some genes such as TIMP3, MLH1, CDH1, RASSF1A, and ESR are often methylated.[10–13] The phenomenon is related to inhibited expression of genes and may affect the development of thyroid tumors through transcriptional regulation of various genes. MSP is a method for rapid detection of methylated CpG sites in CpG island, independent of the restriction enzyme with methylation sensitiveness.[14] It enables to transform non-methylated cytosine into uracil by modifying DNA with sodium bisulfite with the methylated cytosine remaining unchanged and then amplify PCR with methylated and non-methylated primers. It has extremely high sensitivity up to 0.1%, low false positive rate, and less demands of template. Trace DNA come from paraffin embedded tissues, blood, sputum, urine, and thyroid scraping tablets besides fresh tissues. This experiment is to detect aberrant methylation of DAPK gene in PTC by this method. In order to ensure the accuracy and reliability of the results, positive control, negative control, and blank control with water were adopted.

The study found that the methylation rate of DAPK gene in the study group with PTC was 71.4%, which was significantly higher than that in the control group ($\chi^2=19.724$, $P<0.01$). The result suggests that aberrant methylation of DAPK promoter may play an important role in the formation of PTC and may be involved in its development. It is speculated that aberrant methylation in DAPK promoter regions leads to abnormal transcription and down-regulated expression, thereby promoting cell proliferation and

metastasis, inhibiting its apoptosis promoting effect, and eventually leading to PTC.[15,16]

In addition, this study also showed that the methylation of DAPK promoter was not associated with gender, age, tumor size, TNM stage, and thyroid capsular invasion but related to lymph node metastasis. The methylation in DAPK gene promoter regions is relatively higher in patients with PTC accompanied by lymph node metastasis, suggesting that the aberrant methylation may participate in the occurrence, development, and metastasis of the disease.

In order to investigate the relationship of CpG island methylation with gene expression in DAPK promoter, all specimens in the study were given immunohistochemical staining and the results showed that the 58 methylated thyroid tissue cases consisted of 8 with positive DAPK expression and 50 with negative DAPK expression, and that another 62 cases of non-methylated thyroid tissue included 56 with positive DAPK expression and 6 with negative DAPK expression. The results of Spearman's rank correlation analysis showed that methylation of DAPK promoter was negatively correlated with DAPK expression, suggesting that aberrant DAPK methylation may be an important reason for the gene silence and deletion of protein expression. However, there were 6 cases with deletion of protein expression in non-methylated samples, suggesting that DAPK gene silencing may be caused by gene mutation and gene deletion besides gene promoter methylation.

In conclusion, this study provides a molecular biological reference for the diagnosis and prognosis of PTC. Also, it gives certain hints to intervene methylation sites with demethylation agents for prevention and treatment of PTC. However, due to the limitations of time and manpower, the relationship between methylation and prognosis was not explored in this study. Furthermore, the sample size of this experiment was small, so the obtained conclusions need to be further studied and verified. More in-depth research needs to figure out the specific mechanism of methylation as well as the relationships of methylation with tumor and gene transcription inactivation.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

Shandong medical and health science and technology development project 2014WS0457 – "demethylation of thyroid cancer DAPK gene inhibits tumor cell growth."

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