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DAPK Gene Methylation Application in the Early Diagnosis of Nasopharyngeal Cancer

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Abstract: *Background:* Nasopharyngeal cancer is a tumor of high malignancy originating from the epithelial tissues of the nasopharynx, which is often misdiagnosed and missed due to insidious lesions and inconspicuous early symptoms, and is a serious threat to human health. The development mechanism of nasopharyngeal cancer is still not well understood. Death-associated protein kinase (DAPK) is a calcium- and calmodulin- dependent serine and threonine protein kinase, which is involved in the apoptotic process induced by interferon-γ, tumor necrosis factor-α, Fas, etc. and has a pro-apoptotic function. The expression of DAPK in nasopharyngeal cancer tissues was related to the methylation status of CpG islands in the promoter region of its gene. Methylation of DAPK gene may be related to the occurrence and development of nasopharyngeal carcinoma. *Objectives*: The purpose of this article is to investigate the application value of DAPK gene methylation in the early diagnosis of nasopharyngeal cancer. *Method*: Methylation-specific PCR, pyrophosphate sequencing, real-time quantitative PCR and Western Blot test were used to detect the methylation of DAPK gene and its expression status in the blood of patients with nasopharyngeal cancer and chronic rhinopharyngitis. *Result*: There was no significant difference in the methylation rate and expression of DAPK gene in the blood of patients with nasopharyngeal cancer and chronic rhinopharyngitis. *Conclusion*: DAPK gene methylation cannot be used as an indicator for early diagnosis of nasopharyngeal cancer.

Keywords: Nasopharyngeal Cancer, Methylation, Death-Associated Protein Kinase, Polymerase Chain Reaction

1. Introduction

Nasopharyngeal cancer is a tumor of high malignancy originating from the epithelial tissues of the nasopharynx, which is often misdiagnosed and missed due to insidious lesions and inconspicuous early symptoms, and is a serious threat to human health. The development mechanism of nasopharyngeal cancer is still not well understood, and it may be related to various factors such as geographical environment, living habits, genetic inheritance and EB virus infection. In recent years, the study of some tumor-associated genes has received increasing attention, especially the epigenetic regulation of genes has received more attention. The epigenetics of genes refers to heritable changes in gene expression without changes in gene sequence, mainly including gene methylation and histone acetylation, and broadly speaking, epigenetics also includes microRNA. DNA methylation, as an important epigenetic phenomenon, is frequently altered in tumor cells, and hypermethylation of CpG islands of oncogenes is often associated with silencing of gene expression. Several anti-oncogenes have been found to be inactivated by methylation in a variety of tumors. With the advancement of gene methylation studies, gene methylation testing has become a valuable biomarker for early diagnosis and risk assessment of some tumors [1, 2], and more importantly, gene methylation often occurs in the early stages of tumors and can be detected in circulating blood [3].

Death-associated protein kinase (DAPK) is a calcium- and calmodulin- dependent serine and threonine protein kinase [4, 5], which is involved in the apoptotic process induced by interferon-γ, tumor necrosis factor-α, Fas, etc. and has a pro-apoptotic function [6]. Our previous study found that the expression of DAPK in laryngeal and nasopharyngeal cancer tissues was related to the methylation status of CpG islands in

the promoter region of its gene [7, 8], and the present study was intended to investigate the methylation of DAPK gene in the blood of patients with nasopharyngeal cancer to understand its research value in the early diagnosis of nasopharyngeal cancer.

2. Material and Method

2.1. Clinical Data

The blood specimens in this study were obtained from 10 patients with nasopharyngeal cancer and 10 patients with chronic inflammation of the nasopharynx, all of whom were hospitalized from our hospital with suspected nasopharyngeal neoplasia, and all of whom were not treated with radiotherapy or chemotherapy prior to blood sampling. Among the 10 patients with nasopharyngeal cancer, 7 were male and 3 were female, according to the 8th edition of the UICC TNM staging criteria, including T1 stage 2, T2 stage 4, T3 stage 3, T4 stage 1, 6 with lymph node metastasis and 4 without lymph node metastasis.

2.2. Methylation-Specific PCR

After centrifugation of the above blood to isolate the blood cells, the DNA of each group of cells was extracted with DNA extraction kit, and then modified with DNA methylation kit by bisulfite, referring to the sequences of methylation and non-methylation primers of DAPK gene in our previous study. Methylation (M): 5'-GGA TAG TCG GAT CGA GTT AAC GTC-3' (sense) and 5'-CCC TCC CAA ACG CCGA-3' (antisense). Unmethylation (U): 5'-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3' (sense) and 5'-CAA ATC CCT CCC AAA CAC CAA-3' (antisense). The DNA of each group of cells modified with the above sulfite was amplified with two primers respectively, and the annealing temperature was 55°C (methylation), and the above amplified products were sequenced by machine pyrophosphate reaction on a sequencer.

2.3. Fluorescence Quantitative PCR

Total RNA from the above cells was extracted with TRIzol reagent. Reverse transcription was performed with reverse transcriptase to form the first strand of DNA (cDNA). Two pairs of primers, DAPK and β-actin, were designed according to the gene sequences in GeneBank, and PCR was performed using cDNA as template respectively. FES primers: 5'-GCCTGGAGACGGAGAAGAT-3'(S), 5'-AAGTCCCGTGGCTGGTAGA-3' (R); β-actin primers: 5'-GCATGGGTCAGAAGGATTCCT-3' (S), 5'-TCGTCCCAGTTGGTGACGAT-3'. Reaction system: H_2O 6.5μl, Premix EX Taq TM (Probe qPCR) 2× 10μl, Forward primer (10pmol/μl) 0.5μl, Reverse Primer (10pmol/μl) 0.5μl, cDNA 2μl, total volume 20μl. Annealing temperature is 60°C. Relative expression of samples $=2^{-\Delta\Delta CT}$.

2.4. Western Blot

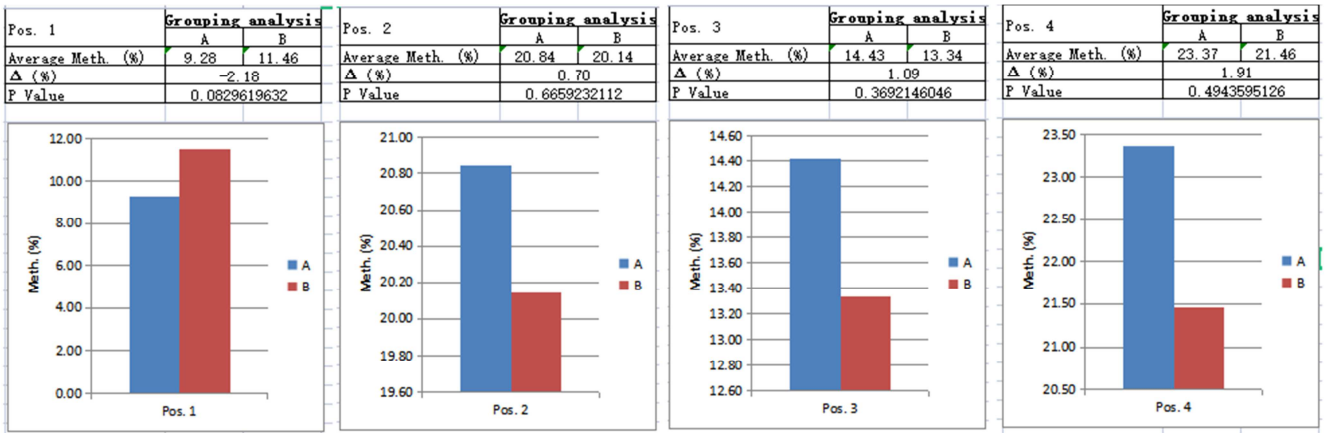
The total protein was extracted from the above cells using the Total Protein Extraction Kit (product of Beijing Pulley Gene Technology Co., Ltd.) (according to the instructions), and the total protein was quantified using the BCA Protein Quantification Kit. Western blotting of protein expression was performed by the following steps: making SDS-PAGE gel, protein loading, electrophoresis, membrane transfer, closure, primary antibody incubation, secondary antibody incubation, color development, digital gel image analysis system photography and analysis of optical density values to record the results.

2.5. Statistical Analysis

SPSS18.0 software was used for statistical analysis of the data, and t-test for independent samples was applied to analyze the differences in the relative expression of FES mRNA and grayscale values of FES protein expression bands. The differences were considered significant if the P value was less than 0.05.

3. Result

The sequencing results of methylation-specific PCR products showed no significant difference in the methylation rate of the eight CpG sites in the promoter region of the DAPK gene between the nasopharyngeal cancer group and the chronic nasopharyngeal inflammatory group (see Figure 1).

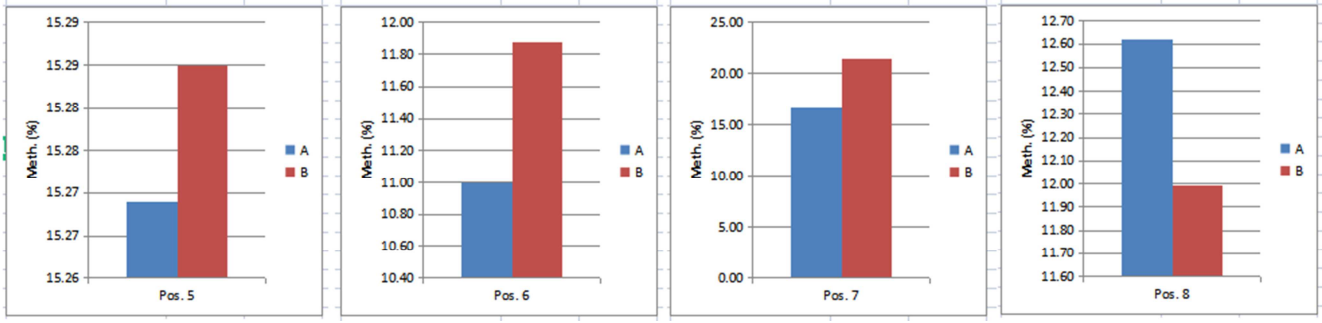


Pos. 5	Grouping analysis	
	A	B
Average Meth. (%)	15.27	15.29
Δ (%)	-0.02	
P Value	0.9952811159	

Pos. 6	Grouping analysis	
	A	B
Average Meth. (%)	11.00	11.87
Δ (%)	-0.87	
P Value	0.6682898381	

Pos. 7	Grouping analysis	
	A	B
Average Meth. (%)	16.66	15.42
Δ (%)	1.24	
P Value	0.6366948426	

Pos. 8	Grouping analysis	
	A	B
Average Meth. (%)	12.62	11.99
Δ (%)	0.62	
P Value	0.8164995737	



A: The nasopharyngeal cancer group; B: The chronic nasopharyngeal inflammatory group

Figure 1. *The methylation rate of the eight CpG sites in the promoter region of the DAPK gene.*

Fluorescence quantitative PCR detected FES mRNA expression, expressed as the relative expression of a single sample, and there was no significant difference between the relative expression of FES mRNA in chronic nasopharyngitis group (2.47±0.31) and nasopharyngeal cancer group (2.45±0.33), t=0.156, P>0.05, the difference was not statistically significant.

The mean optical density of protein bands in the nasopharyngeal cancer group (263.1±79.5) was not significantly different from that in the nasopharyngitis group (292.4±120.2) by Western Blot (see Figure 2), t=0.645, P=0.528, and the difference was not statistically significant.

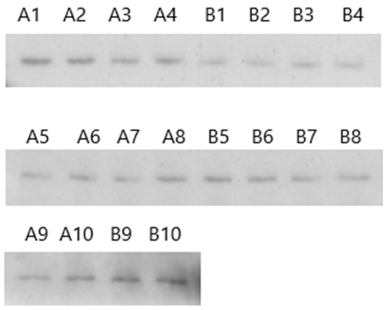


Figure 2. *The expression of DAPK protein in the nasopharyngeal cancer group and the nasopharyngitis group.*

A: The nasopharyngeal cancer group; B: The chronic nasopharyngeal inflammatory group

4. Discussion

Nasopharyngeal cancer is a common malignant tumor in otolaryngology, especially in the Guangdong region of China, which is a high incidence area in the world, so nasopharyngeal cancer is called "Guangdong cancer". Although targeted therapy has improved the cure rate of some patients with nasopharyngeal cancer in recent years, the survival rate and quality of survival of patients with nasopharyngeal cancer have not been significantly improved. To improve the survival rate and survival quality of nasopharyngeal cancer patients, the early diagnosis rate of nasopharyngeal cancer patients should be improved and personalized treatment should be given.

Huang [9] found that Death-associated protein kinase 1 suppresses hepatocellular carcinoma cell migration and invasion by upregulation of DEAD-box helicase 20. Zhai [10] discover that Reduced expression levels of the death-associated protein kinase and E-cadherin are correlated with the development of esophageal squamous cell carcinoma. Zhang [11] found that Diagnostic potential of methylated DAPK in brushing samples of nasopharyngeal carcinoma. Zhao [12] think that methylation rate of DAPK gene is higher in untreated ALL patients than in AML patients and normal subjects. DAPK gene methylation is not correlated with the clinical features of ALL patients but is probably related with the low gene expression level of DAPK in these patients.

In this study we examined the methylation of DAPK gene, gene and protein expression in the blood of patients with nasopharyngeal cancer. We found that there was no significant difference in the methylation of DAPK gene in the blood of patients with nasopharyngeal cancer and chronic rhinopharyngitis, as well as there was no significant difference in either DAPK gene expression or protein expression between the two groups. Our previous study found that the rate of DAPK gene methylation in nasopharyngeal cancer tissues was 76.1%, and the gene expression was significantly lower than that in chronic inflammatory tissues of the nasopharynx [7]. These results suggest that most of the nasopharyngeal cancer patients in the study group did not have cancer cells in the blood circulation.

Nasopharyngeal cancer is a tumor of high malignancy originating from the epithelial tissues of the nasopharynx. Because insidious lesions and inconspicuous early symptoms, it is often seen as a neck mass and the primary focus of nasopharynx found only after cytological puncture for metastatic cancer. Early metastasis of nasopharyngeal cancer is mainly lymphatic metastasis and less blood metastasis, and cancer cells rarely metastasize distantly through blood, which is the reason why we detected no difference in methylation and expression of DAPK gene in blood of nasopharyngeal cancer patients and chronic nasopharyngitis patients.

In conclusion, lymphatic metastasis is predominant in the early stage of nasopharyngeal cancer, and less cancer cells

enter the blood circulation. The detection of DAPK gene methylation in blood cannot be used for the early diagnosis of nasopharyngeal cancer.

Conflicts of Interest

The authors report no conflict of interest.

Acknowledgements

Song Zhang designed the study and supervised all experiments. Hong-Yan Yuan executed RT-PCR and Western Blot experiments, and drafted this paper. Jia-Hui Tang executed data sorting. All authors read and approved the final manuscript.

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