## Original Article
# Methylation of DAPK, P16 and E-Cadherin gene in liver cancer tissues and protein expression level

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**Abstract:** As the most common mechanism of epigenetics, DNA methylation has not been fully illustrated in tumor disease. Hepatocellular carcinoma (HCC) is one common malignant tumor but with limited epigenetic knowledge about its molecular mechanism. This study thus mainly investigated the methylation status at promoter regions of death associated protein kinase (DAPK), poly tumor suppressor gene (P16) and E-Cadherin, along with protein expression levels. Paraffin sections of HCC or liver cirrhosis tissues were tested for promoter methylation condition of DAPK, P16 and E-cadherin genes using methylation specific PCR (MSP) approach. Immunohistochemistry (IHC) staining was applied to detect protein expression levels for statistical analysis. MSP assay showed methylation positive rates of DAPK, P16 and E-Cadherin genes at 69.23%, 79.49% and 58.97%, respectively, in HCC tissues. All of these rates were significantly higher than non-tumor tissues (28.21%, 2.56% and 25.64%, P<0.05). P16 gene methylation rate in cirrhosis tissues was lower than HCC group (P<0.01). While DAPK and E-Cadherin gene had not significantly different of methylation rate (P>0.05). IHC staining revealed significant correlation between methylation and protein expression level of those genes (P<0.05), as positive methylation HCC cells had lower protein levels than non-methylation group. Hyper-methylation existed in promoter of DAPK, P16 and E-Cadherin genes in HCC tissues, providing further evidences for early diagnosis of HCC.

**Keywords:** Hepatocellular carcinoma, death associated protein kinase, P16, E-Cadherin, gene methylation

## Introduction

Hepatocellular carcinoma (HCC) is one common malignant tumor worldwide, and is the third leading cause for death of tumors [1, 2]. Its detailed pathogenesis mechanism, however, requires further study. Currently epigenetics has become one critical mechanism regulating tumor pathogenesis drawing research interests. Recent studies recognized methylation of gene promoter region as one important reason for inactivation of tumor suppressor genes, for mediating cell apoptosis, cell cycle regulation, DNA repair, message transduction and Wnt signaling pathway, in addition to close correlation between DNA methylation and tumor occurrence or pathological types [3, 4]. DNA methylation has been implicated in various cancers including colorectal cancer, esophagus carcinoma and pulmonary cancer [5-7]. Meanwhile, DNA methylation can work as potential tumor markers [8, 9] for studying related diseases.

The role of abnormal methylation of gene promoter in HCC, however, requires further studies. This study utilized methylation specific PCR (MSP) technique, to detect methylation status of gene promoter regions of death associated protein kinase (DAPK), P16 and E-Cadherin genes in HCC and non-tumor liver tissues, in addition to protein expression level of those genes, in order to reveal the role of DNA methylation in early diagnosis and treatment of HCC.

## Materials and methods

### General information

A total of 72 tissues samples were collected from liver cancer or liver cirrhosis patients between August 2014 and July 2015 in the Affiliated Hospital of Weifang Medical University. Among those there were 39 liver cancer patients (31 males and 8 females, average age = 61.0 ± 8.29 years) whose tumor and adjacent

**Table 1.** Methylation (M) and un-methylation (U) primer sequence

| Target gene | Sense strand (5'-3') | Anti-sense strand (5'-3') | Product size (bp) |
|---|---|---|---|
| DAPK (M) | GGATAGTCGGATCGAGTTAACGTC | TAACTAAAAATTCACCTACCGAC | 98 |
| DAPK (U) | GGAGGATAGTTGGATTGAGTTAATGTT | CACAACCAATCAACAACACA | 106 |
| P16 (M) | TTATTAGAGGGTGGGGTGGATTGT | CAACCCCAAACCACAACCATAA | 150 |
| P16 (U) | TTATTAGAGGGTGGGGCGGATCGC | GACCCCGAACCGCGACCGTAA | 151 |
| E-cadherin (M) | TTAGGTTAGAGGGTTATCGCGT | TAACTAAAAATTCACCTACCGAC | 116 |
| E-cadherin (U) | TAATTTTAGGTTAGAGGGTTATTGT | CACAACCAATCAACAACACA | 97 |

**Table 2.** Promoter methylation level of DAPK, P16 and E-cadherin genes

| Group | N | DAPK methylation | | P16 methylation | | E-cadherin methylation | |
|---|---|---|---|---|---|---|---|
| | | +(n) | -(n) | +(n) | -(n) | +(n) | -(n) |
| Liver cancer | 39 | 27 | 12 | 31 | 8 | 23 | 16 |
| Adjacent | 39 | 11 | 28 | 1 | 38 | 10 | 29 |
| Liver cirrhosis | 33 | 26 | 7 | 6 | 27 | 17 | 16 |

Note: n represents number of cases.

tissue samples were collected. Another 33 liver cirrhosis patients (20 males and 13 females, average age = 58.27 ± 6.81 years) were also recruited. No significant difference has been found regarding ages. All patients had confirmed diagnosis by pathological examinations, and with complete medical history. This study has been approved by the ethical committee and is carried out under written consents from patients. All tissues were collected and kept in liquid nitrogen.

*MSP primer*

The methylation/un-methylation primer sequence of DAPK, P16 and E-cadherin genes were designed as previously documented [10-12]. Sequences were shown in **Table 1**.

*DNA extraction and bisulfite modification*

Total DNA was extracted from tissues using Wizard Genomic DNA Purification Kit (Promega, US) following manual instruction. DNA was quantified in UV spectrometer and was kept under -80°C. Bisulfite modification and purification was carried out using phenol and sodium bisulfite (Sigma, US) as previously documented [3].

*Methylation specific PCR*

MSP was carried out in a 30 μL system consisting of 15 μL PCR mixture, 1 μL primers, 1~4 μL modified DNA and ddH$_2$O. PCR conditions were: 94°C pre-denature for 5 min, followed by 40 cycles each containing 94°C denature for 45 sec, 60°C annealing for 45 sec, and 72°C elongation for 45 sec, and ended with 72°C elongation for 7 min. Amplification products were separated by 2% agarose gel electrophoresis and were imaged under an automatic gel imaging system. Those samples had positive reaction using MF/MR specific primers were positive for methylation while those had only positive reaction using UF/UF primers were methylation negative.

*Immunohistochemistry*

Tissue sections were baked in 70°C incubator for 20~30 min, and were dewaxed by xylene and gradient ethanol. After hydration, antigen retrieval was performed in heated citric acid buffer (pH 6.0) for 90 sec. Endogenous peroxidase activity was quenched by 3% H$_2$O$_2$ in methanol, followed by blocking in normal goat serum. Primary antibody was added for 4°C overnight incubation. Secondary antibody and development was performed using IHC test kit (Zhongshan, China). Cell nucleus was counterstained by hematoxylin for 40 sec, followed by HCl-ethanol treatment, dehydration and coverslip mounting. Under a microscope, brown granules in cytoplasm were deduced as the positive staining. A semi-quantitative analysis was performed as previously established [4]. High power (×200) images were captured, staining score was given by the sum of intensity score (0, no staining; 1, light yellow; 2, dark yellow; 3, dark brown) and positive percentage score (<5%, 0; 5%~25%, 1; 26%~50%, 2; 51%~75%, 3; >75%, 4). Total score was interpreted as negative (0), weak positive (1+, 1~2), positive (2+, 3~5) or strong positive (3+, 6~7).

*Statistical analysis*

SPSS 16.0 software was used to process all collected data, of which measurement data
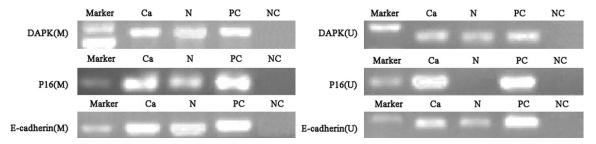
**Figure 1.** MSP bands showing promoter methylation of DAPK (upper), P16 (middle) and E-cadherin (lower) genes. M, methylated alleles; U, un-methylated alleles; Ca, liver cancer; N, non-tumor tissues; PC, methylated/un-methylated DNA positive control; NC, negative control.

**Table 3.** Methylation status of DAPK, P16 and E-cadherin gene and protein expression

| Methylation status | | Protein expression | | *P* value |
|---|---|---|---|---|
| | | +(n) | -(n) | |
| DAPK | Methylated | 5 | 22 | 0.013 |
| | Un-methylated | 7 | 5 | |
| P16 | Methylated | 3 | 28 | 0.004 |
| | Un-methylated | 5 | 3 | |
| E-cadherin | Methylated | 4 | 19 | 0.002 |
| | Un-methylated | 11 | 5 | |

Note: n represents number of cases.

were presented as mean ± standard deviation (SD) while enumeration data were compared by chi-square test. A statistical significance was defined as P<0.05.

## Results

*Methylation level of promoters*

MSP method was used to detect the methylation status of gene promoters of DAPK, P16 and E-cadherin genes. We found the methylation positive percentage as 69.23% (27/39), 79.49% (31/39) and 58.97% (23/39) in liver cancer tissues for DAPK, P16 and E-cadherin genes, respectively. Such figures in liver cirrhosis tissues were 78.79% (26/33), 18.18% (6/33) and 51.52% (17/33), while in adjacent tissues were 28.21% (11/39), 2.56% (1/39) and 25.64% (10/39). Between-group comparison revealed lower methylation level of P16 genes in cirrhosis group compared to liver cancer tissues (P<0.01), while no significant difference existed between DAPK and E-cadherin genes (P>0.05). The methylation level of all three genes was significantly higher in liver cancer tissues compared to adjacent tissues (P<

0.01). Detailed results were shown in **Table 2** and **Figure 1**. In addition, no significant difference of methylation level has been discovered regarding clinical/pathological features.

*Gene methylation status and protein expression*

Based on the methylation status, we further analyzed protein expression level of DAPK, P16 and E-cadherin level in liver cancer tissues using IHC methods. As shown in **Table 3**, gene methylation level of DAPK, P16 and E-cadherin genes were negatively correlated with their protein expression levels (P<0.05). Protein expression level was decreased in hyper-methylation genes. Those liver cancer tissues with methylation positive status had lower protein level than un-methylated counterparts (**Figure 2**).

## Discussion

Tumor pathogenesis is one progressive process in which both genetic and epigenetic mechanisms lead to the activation of oncogenes and inactivation of tumor suppressor genes, plus the stimulus from exogenous factors [13]. Epigenetic is a type of gene expression regulation in which DNA sequence keeps intact. In tumor patients, it is often observed that epigenetic gene silencing and consequent inactivation of tumor suppressor genes. The methylation of promoter region of tumor suppressor genes is thus believed as one important epigenetic mechanism underlying liver cancer, and may provide new insights for searching novel biomarkers of liver cancer diagnosis [14, 15], has recently drawn lots of research interests. Currently the methylation of promoter region of DAPK, P16 and E-cadherin genes has been recognized, while further in-
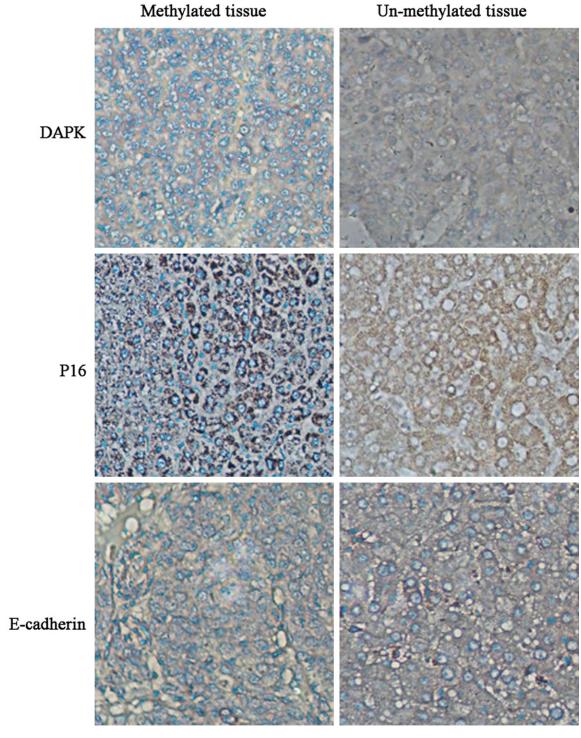
**Figure 2.** DAPK, P16 and E-cadherin protein expression level by IHC staining.

depth studies are required. This study utilized MSP approach to detect the methylation status of DAPK, P16 and E-cadherin genes, in addition to analyzing protein expression levels of those genes, in an attempt to find liver cancer related methylation markers.

DAPK is one serine/threonine kinase participating in various cellular processes including cell apoptosis, autophage and inflammation [16]. Study has shown the correlation between DAPK and various diseases including cancer, neuronal death and stroke [17], as it can par-

ticipate in tumor cell metastasis for aggravation of disease. Related studies have demonstrated the alternation of expression and function of DAPK in cancer patients [18, 19]. Its methylation phenomena are likely to be related with lymph node metastasis. Cell cyclin dependent kinase inhibitor P16, also named as mutli-tumor suppressor gene, belongs to INK4 family and participates in cell cycle control. It often display mutation, truncation, and abnormal methylation in tumor cells, leading to the inactivation of gene expression, further causing accelerated cell proliferation and shortening of cell cycles. It is the most common tumor-related abnormal methylation gene and can be detected even at early stage of tumors [20]. Studies believed that P16 might participate in tumor related pathways in conjunction with DAPK [21]. E-cadherin can regulate calcium-dependent cell adhesion in various epithelial tissues, as its malfunction could interrupt the interaction between cells. The mutation of E-cadherin in tumor patients may cause abnormal detachment and mobility of cells, further strengthening cell invasion and infiltration/metastasis potency [22]. Previous study has shown the down-regulation of E-cadherin gene in tumor tissues by promoter methylation, thus inducing unfavorable prognosis of patients [23, 24].

This study utilized a methylation specific PCR approach, to detect methylation status of DAPK, P16 and E-cadherin gene promoters in liver cancer and non-tumor tissues. Results showed that liver cancer tissues had significantly higher positive rate of gene methylation compared to adjacent non-tumor tissues, indicating the alternation of epigenetics in liver cancer. Such methylation may frequently occur in liver cancer pathogenesis. The methylation rate of P16 gene in liver cirrhosis was relatively lower than liver cancer group, while no statistically significant difference existed between these two tissues regarding methylation rate of DAPK and E-cadherin gene, suggesting the possible relationship between DAPK and E-cadherin with liver cirrhosis. In addition, IHC staining revealed significant correlation between methylation status of DAPK, P16 and E-cadherin genes and their protein levels. Those liver cancer tissues with positive for methylation had lower protein expression level than un-methylated groups. These results collectively suggest-

ed the participation abnormal methylation of promoter regions of those three genes in the pathogenesis of liver cancer via modulating protein expression, as consistent with previous reports [25]. No significant correlation, however, existed between gene methylation and clinical features, probably due to relatively small sample size in this study.

In summary, promote methylation of tumor related genes plays a crucial role during the occurrence and progression of cancers. This study indicated that abnormal methylation of DAPK, P16 and E-cadherin genes might be important molecular events during early stage liver cancer. These genes participate in occurrence and progression of liver cancer. Abnormal methylation of promoters of these genes largely affects protein expression, providing new insights for investigating the mechanism of liver cancer pathogenesis. This study paved grounds for developing early molecular marker for diagnosis of liver cancer, although further large-sample and large-scale screening of gene promoter methylation are required.

**Disclosure of conflict of interest**

None.

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