Basic Research Paper

Promoter methylation of DAPK gene in cervical carcinoma

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Background and Objective: Aberrant DNA methylation plays important roles during multistage carcinogenesis in various human organs. This study was to explore the relationship between the promoter methylation and inactivation of DAPK gene in cervical cancer. Methods: The promoter methylation of DAPK was investigated with methylation-specific polymerase chain reaction (MSP) in 52 specimens of cervical cancer, 60 specimens of cervical intraepithelial neoplasia (CIN) and 20 specimens of normal cervical squamous epithelial tissues. Its correlation to clinicopathologic features of cervical cancer was analyzed. The protein expression of DAPK was detected by immunohistochemistry. Results: The methylation rate of DAPK gene promoter was significantly higher in cervical cancer tissues than in CIN (65.4% vs. 18.3%, p < 0.05); while no methylation of DAPK gene was found in normal cervical tissues. The methylation rate of DAPK gene was significantly higher in cervical squamous cell carcinomas than in adenocarcinomas (80.0% vs. 16.7%, p < 0.001). Promoter methylation of DAPK was negatively correlated to its protein expression (r = -0.849, p < 0.001). Conclusion: The promoter methylation may lead to inactivation of DAPK gene, and may be related with tumorigenesis of cervical cancer.

Cervical cancer is an important cause of death in women worldwide. There is a strong association between certain subtypes (high risk) of human papillomavirus (HPV) and cervical cancer. However, other factors are also involved in cervical carcinogenesis, because the majority of patients with HPV infection will not develop invasive cervical cancer. Methylation of CpG islands in promoter regions were found to be a major mechanism of inactivation of tumor suppressor genes or cancer-related genes in human cancer. Gene expression is frequently lost in tumors due to methylation of the gene. Death-associated protein kinase (DAPK) is a novel 160 ku calmodulin-dependent serine/threonine kinase operating as a positive mediator of apoptosis, while apoptosis links to the development, progression, and metastasis of human cancer. DAPK participates in interferon-γ (INFγ), Fas, tumor necrosis factor-β (TNFβ), Ceramide, ERK, C-myc and E2F-induced apoptosis, which suggests that DAPK is a conjunction of all kinds of apoptosis signals. DAPK promoter methylation involving in many primary tumors is a research hot spot. In this study, we had detected the status of promoter methylation and protein expression of DAPK gene in cervical cancers and cervical precancerous lesions to explore the correlation of DAPK protein expression to the development of cervical carcinoma.

Materials and Methods

Specimens and subject data. Specimens were obtained from 52 primary cervical carcinoma patients and 60 cervical intraepithelial neoplasia (CIN) patients who had received operation during 2005–2007 at Gynecological Department of the First Affiliated Hospital of Zhengzhou University. All diagnoses were confirmed by pathologic examination. The cervical carcinoma patients were aged of 25–71 years with a median of 42 years, and none of them had received radiotherapy or chemotherapy before operation. According to the 1995 staging system of the International Federation of Gynecology and Obstetrics, 18 patients were at stage I, 20 at stage II, nine at stage III, and five at stage IV. Histologically, 40 had squamous cell carcinoma (SCC) and 12 had adenocarcinoma (AC); 11 had well differentiated tumors; 19 had moderately differentiated tumors, and 22 had poorly differentiated tumors; Eight had lymph node metastasis. Of the 60 CIN patients, 20 had CINI, 20 had CINII, and 20 had CINIII. In addition, 20 specimens of normal cervical squamous epithelia obtained from uterine leiomyoma patients were used as control. Each sample was divided into two portions: one portion was immediately frozen and stored at -80˚C; the other was fixed in buffered formalin embodied in paraffin for routine pathologic examination and immunohistochemistry. All samples were examined by two pathologists.

Reagents and instruments. Genomic DNA Extraction Reagent Kit was bought from Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. Hydroquinone and sodium bisulfite were bought from Sigma Company. Wizard DNA Purification Reagent Kit was bought from Promega Company. DNA Ladder Marker and TaqTM Hot Start Version were bought from TaKaRa Company. Primers of DAPK gene were synthesized by Beijing Augt Company. DAPK rabbit anti-human polyclonal antibody was bought from BD bioscience Company. Streptavidin-peroxidase (SP)
Reagent Kit and DAB solution were bought from Beijing Zhong Shan-Golden Bridge Technology Co. LTD. Centrifuge was bought from Heraeus Company. UV-visible spectrophotometer was bought from Startorius Company. Instrument for polymerase chain reaction (PCR) was bought from Biometra Company. DYY-III Electrophoresis System was bought from Beijing Liu Yi Instrument Factory. UV illumination was bought from Synoptics LTD Company.

**DNA extraction and sodium bisulfite modification.** Genomic DNA extraction was performed according to the reagent kit protocol. A total of 1–2 μg genomic DNA was diluted to 50 μL with distilled H2O, denatured in 5.5 μL 3 mol/L NaOH at 42˚C for 30 min, then added with 30 μL 10 mmol/L hydroquinone and 520 μL 3.6 mol/L sodium bisulfite, and incubated at 50˚C for 16 h under mineral oil. Modified DNA was purified using Wizard DNA Purification System. After ethanol precipitation, samples were dissolved in 20 μL water and stored at -20˚C until use.

**Methylation-specific PCR (MSP).** The promoter methylation of DAPK was investigated with methylation-specific polymerase chain reaction (MSP). Primer sequences of DAPK were 5'-GGA TAG TCG GAT CGA GTT AAC GTC-3' (sense) and 5'-CCC TCCCAA ACG CCGA-3' (antisense) for the methylated reaction and 5'-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3' (sense) and 5'-CAA ATC CCT CCC AAA CAC CAAGA-3' (antisense) for the unmethylated reaction, which generated PCR products of 98 bp and 106 bp, respectively. Step-down PCR reactions were performed in a 25 μL reaction system containing 2 μL bisulfite-modified DNA, 2 μL 10 × PCR buffer, 4 μL 2.5 mmol/L dNTP Mixture, 0.3 μL hot start Taq-polymerase, 1 μL of each primer, and 14.7 μL H2O. PCR conditions were 95˚C for 5 min, 35 cycles of 94˚C for 45 s, annealing at 60˚C for 45 s and 72˚C for 45 s, followed by a final extension step at 70˚C for 7 min. DNA extracted from peripheral lymphocytes of healthy subjects was used as unmethylated positive control; placenta DNA digested with Sss I methyltransferase was used as methylated positive control; H2O was used as a blank control.

**Immunohistochemistry.** Immunohistochemistry for DAPK protein was performed with DAPK antibody (dilution 1:200) using Diaminobenzidine tetrahydrochloride Substrate Kit. Positive and negative control slides were run in parallel with test slides. The cytoplasm of positive cells was stained in yellow.

**Data analysis.** The differences of positive rates of DAPK methylation and protein expression among groups were analyzed by χ² test, and the correlation between two variables was examined by Spearman rank-correlation test using SPSS 13.0 software. A p value of < 0.05 was considered as significant.

**Results**

**Frequency of DAPK promoter methylation.** There was an increasing trend of DAPK promoter methylation among normal cervical squamous epithelial tissues, CIN and cervical cancer tissues (Fig.1), with methylation rates of 0% (0/20), 18.3% (11/60) and 65.4% (34/52), respectively (χ² = 39.639, p < 0.001). There was no significant difference in the methylation rate of DAPK promoter between CIN and normal cervical squamous epithelial tissues (χ² = 2.846, p > 0.05), while the difference between cervical cancer tissues and normal cervical squamous epithelial tissues was significant (χ² = 24.777, p < 0.001), and the difference between cervical cancer tissues and CIN was also significant (χ² = 25.658, p < 0.001).

**Correlations of DAPK promoter methylation to clinicopathologic features of cervical cancer.** The methylation rate of DAPK promoter was significantly higher in cervical squamous cell carcinoma than in cervical adenocarcinoma (p < 0.001), but no significant differences were found among subgroups stratified according to clinical stage, differentiation, and lymph node metastasis (p > 0.05) (Table 1).

**Correlation of DAPK promoter methylation to its protein expression.** DAPK protein was expressed in cytoplasm of cervical cancer cells (Fig. 2). DAPK protein was detected in four of 45 methylated cervical samples, but in 82 of 87 unmethylation samples. DAPK promoter methylation was negatively correlated to its protein expression (r = -0.849, p < 0.001).

**Discussion**

In addition to HPV infection, other factors are also involved in cervical carcinogenesis. Recently, the inactivation of tumor suppressor genes was paid more attention to. Hypermethylation of CpG islands in the promoter regions of tumor suppressor genes results in transcriptional repression and loss of protein expression.
Methylation of various tumor-related genes were detected in cervical cancer, such as RASSF1A, CAGE, CDH1, RARB, HIC1, APC, MGMT, p16, DAPK, and so on.

MSP is a simple rapid and inexpensive method to determine the methylation status of CpG islands independent of methylation-sensitive restrictive endonuclease. In MSP, unmethylated cytosine will be transformed into uracil by sodium bisulfite modification, while methylated cytosine will retain, then primers for methylated and unmethylated DNA will be used in PCR. Extraordinary sensitivity of 0.1% can be achieved in MSP, with a low false positive rate. MSP also allows the analysis of very small samples, including fresh tissues, paraffin-embedded tissues, serum, sputum, urine, cervical scrapes, and so on.6, 7 In this study, MSP was used to detect the status of methylation of DAPK gene promoter. To guarantee the results’ accuracy, we ran positive, negative and blank controls in parallel with experiment.

We found that the methylation rate of DAPK gene was 65.4% in cervical cancer samples, while no methylation was found in normal cervical squamous epithelial tissues. This suggests that the methylation of DAPK promoter is a tumor-specific phenomenon. We had also detected DAPK gene methylation in 18.3% of the CIN tissues, indicating it as an early event in cervical carcinogenesis and it may play an important role in tumor development. RASSF1A gene methylation does not exist in normal cervical squamous epithelial tissues, which is similar to the satus of DAPK gene. But not all tumor suppressor genes have the same situation. As Lai et al.8 reported, the methylation rate of BLU was 20% in CINI tissues, 12.5% in normal cervical tissues. From normal cervical tissues, CIN to cervical cancer tissues, the methylation rates of most tumor suppressor genes are increased.

In our study, the methylation rate of DAPK was significantly higher in cervical squamous cell carcinoma than in cervical adenocarcinoma (p < 0.001). This result is coincided to previous report.9 The association between methylation and pathologic type suggests different methylation patterns of DAPK in cervical squamous cell carcinoma and cervical adenocarcinoma. The methylation rates of CDH1, RARB, FHIT, and MGMT are higher in cervical squamous cell carcinoma than in cervical adenocarcinoma, while those of HIC-1 and APC are higher in cervical adenocarcinoma than in cervical squamous cell carcinoma.10 The methylation patterns of various genes in cervical cancer are different. We also found that there were no significant differences in the methylation rate of DAPK between subgroups of cervical cancer stratified by clinical stage, differentiation, and lymph node metastasis (p > 0.05), which suggests that the methylation of DAPK is an early event in cervical carcinogenesis. According to previous research, DAPK promoter methylation might be an indicator of tumor recurrence: the 2-year recurrence rates were 80.0% in superficial bladder cancer patients with DAPK promoter methylation and 28.0% in the patients without DAPK promoter methylation.11 DAPK promoter methylation might also be a useful prognostic marker of tumors: the mean survival after initial treatment was significantly shorter in T-cell leukemia patients with DAPK promoter methylation than in those without DAPK promoter methylation (49.3 months vs. 133.2 months, p < 0.05).12 Researchers have tried several methods to re-express DAPK protein by DAPK promoter demethylation in human cancer cells and intend to cure tumors.

In our study, DAPK protein expression was detected in 4 of 45 cervical samples with DAPK promoter methylation and 82 of 87 cervical samples without DAPK promoter methylation. DAPK promoter methylation is negatively correlated to its protein expression, suggesting that CpG islands methylation is an important cause of DAPK gene silence and loss of protein expression. However, DAPK protein expression was also absent in 5 samples without DAPK promoter methylation. These results support that other mechanisms, such as gene mutation and loss of heterozygosity (LOH), are involved in DAPK gene silence besides promoter methylation.

In this study, we have demonstrated that DAPK promoter methylation is a frequent epigenetic event in cervical carcinoma, indicating DAPK promoter methylation as an important mechanism of DAPK gene inactivation which may participate in cervical carcinogenesis.

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