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Detection of DKK-1 Gene Methylation in Exfoliated Cells of Cervical Squamous Cell Carcinoma and its Relationship with High Risk HPV Infection

He Yuan-Hada[1], Su Rui-jun[2] and Jian Zheng[3*]

[1]Basic Medical College of Inner Mongolia Medical University, PR China

[2]Clinical Laboratory of Affiliated Hospital of Inner Mongolia Medical University, PR China

[3]Department of Gynecology and Obstetrics, The Affiliated Hospital of Inner Mongolia Medical University, PR China

Abstract

Purpose: *To detect the methylation of Dickkopf-associated protein 1 (DKK-1) gene promoter in cervical exfoliated cells and to study its clinical significance in cervical squamous cell carcinoma （CSCC） and its relationship with high risk HPV infection.*

Methods: *Methylation-specific PCR (MSP) was utilized to detect the methylation of DKK-1 gene promoter in cervical exfoliated cells from 40 patients with CSCC and 40 patients with chronic cervicitis in the Affiliated Hospital of Inner Mongolia Medical University. The methylation rate of DKK-1 gene promoter in different clinicopathological factors and its relationship with high-risk HPV infection was compared, and different detection methods were compared.*

Result: *The degree of methylation of DKK-1 gene promoter in CSCC group was significantly higher than that in cervicitis group ($P < 0.05$). In CSCC group, the degree of methylation was significantly different in high risk HPV infection, histological differentiation, tumor size, lymph node metastasis and the International Federation of Gynecology and Obstetrics (FIGO) staging (all $P < 0.05$). The degree of methylation is not related to the type of high risk HPV infection ($P > 0.05$). The sensitivity, specificity and accuracy of DKK-1 gene methylation combined with high risk HPV detection were 96.7%, 78.0% and 85.0%, respectively.*

Conclusion: *Methylation of DKK-1 gene promoter in cervical exfoliated cells of patients with CSCC is related to high-risk HPV infection and different clinicopathological factors, but the degree of methylation of DKK-1 gene is not related to the type of high-risk HPV infection. It can be used to estimate the development and prognosis of CSCC and provide clues for targeted treatment of CSCC. DKK-1 gene methylation combined with high risk HPV detection can improve the sensitivity of CSCC diagnosis,and has high specificity and accuracy, which may become a non-invasive screening method for CSCC.*

Keywords: *cervical squamous cell carcinoma; DKK-1·Methylation*

Introduction

Cervical cancer is the fourth leading gynecologic malignancy in the world with high incidence rate and high mortality rate. Each year, there are about 570000 new cases and more than 310000 death cases [1]. Studies have shown that high risk human papillomavirus (HPV) infection is the main cause of cervical cancer [2]. High risk HPV16, 18 or 31 were detected in 99.7% of cervical squamous cell carcinoma [3]. At present, cervical cancer screening is mainly carried out by high risk HPV detection and liquid-based cytology in the world, but the sensitivity and specificity of these methods are still limited [1]. After infection with HPV, only less than5% of patients will develop into cervical cancer, so it is speculated that there may be other factors involved in the occurrence and development of cervical cancer. DNA methylation is one of the earliest epigenetic modification methods. It can change the genetic performance without changing the DNA sequence. It is an exogenetic mechanism. It can be used as a biomarker and prognosis evaluation index for early diagnosis of tumors. It has important significance for tumor screening and risk assessment, early diagnosis, stage classification, prognosis judgment and treatment monitoring. Methylation of tumor suppressor genes plays an important role in the development of cervical cancer. DKK-1 is a tumor suppressor gene found in recent years. As an inhibitor of the Wnt signaling pathway, DKK-1 has abnormal expression in cervical squamous cell carcinoma tissues and serum [4,5]. Methylation of DKK-1 gene promoter also exists in cervical squamous cell carcinoma [6]. The detection of cervical exfoliated cells is the first step in the clinical screening of cervical lesions, which is more noninvasive, convenient and quick than the cervical histopathological examination, and easy for patients to accept, which is beyond the

reach of tissue biopsy. This study examined the relationship between the methylation of DKK-1 gene promoter and high risk HPV infection in cervical squamous cell carcinoma exfoliated cells, so as to determine its clinical value in non-invasive screening, pathological degree and prognosis evaluation of cervical squamous cell carcinoma.

Materials and Methods

Tissue collection

From January 2018 to April 2019, patients with cervical squamous cell carcinoma admitted to the Affiliated Hospital of Inner Mongolia Medical University were selected as the study object. Inclusion criteria: (1) primary patients, who had not received chemotherapy or surgery before; 2) confirmed by clinical manifestations, physical examination, imaging and histopathological examination; 3) excluded patients with severe cardiac cerebral lung and other tumors. A total of 40 patients were enrolled into the experimental group, with an average age of (53.0 ± 2.0) years; the pathological types were cervical squamous cell carcinoma; according to the FIGO standard in 2014, the staging included 14 cases in stage I, 21 cases in stage II, 4 cases in stage III and 1 case in stage IV. In addition, 40 cases of chronic cervicitis in the same period were taken as the control group, with an average age of (45.9 ± 4.4) years. There was no significant differences in age between the experimental group and the control group ($P > 0.05$). Then, use the cervical exfoliated cell collector (Sub energy biotechnology Co.,Ltd,Shenzhen,China)to brush the cervix at the junction of the external cervix and the cervical canal of the above patients, collect the cervical exfoliated cells, put the collector into the matching tube, shake it gently, and wash the cell samples on the collector in the solution for preservation.

Methylation detection of DKK-1 gene promoter

According to the instructions of DNA extraction kit(Tiangen biochemical technology Co.,Ltd, Beijing,China), the total DNA of cervical exfoliated cells in experimental group and control group was extracted. The promoter methylation of DKK-1 gene was detected by MSP method. The DNA was modified by sodium bisulfite (Sigma Aldrich Trading Co.,Ltd, Shanghai,,China)and then recovered by recovery kit((Tiangen biochemical technology Co.,Ltd, Beijing,China). Methylated primers were designed by methprimer 2.0 primerdesign (Biotechnology Co., Ltd ,Shanghai, China). The primer sequence is shown (Tab 1). Using the methylated PCR kit takaraepitaq ™ HS (Baori Medical Biotechnology Co., Ltd, Beijing,China) to operate methylation PCR.The PCR reaction system was 25μL and the circulation condition was 95℃. Pre denaturing for 5min, 94℃ denaturing for 30min, 64℃ annealing for 30s, 72℃ extending for 30s, 35 cycles in total, and the last 72 ℃ extending for 10min. PCR products were detected by agarose gel electrophoresis. After photographing, the gray value of each strip was measured, and the gray value of the target gene strip was compared with that of the internal reference strip. Repeat three times for each sample and take the mean value (Table 1).

HPV typing test procedure

Step 1: Extract HPV DNA from sample cells(Tiangen biochemical technology Co.,Ltd, Beijing,China). Step 2: PCR amplification by PE9600 PCR amplification instrument(MBI company,USA). Step 3: The nucleic acid molecules were hybridized rapidly by HybriMax medical nucleic acid molecular rapid hybridization instrument(Kaipu Biotechnology Co., Ltd, Guangdong,China). Step 4: result judgment: according to the distribution of HPV subtypes on the chip by HPV nucleic acid amplification and typing test kit(Kaipu Biotechnology Co., Ltd, Guangdong,China), the corresponding coloring points are used to determine the HPV subtypes. Each chip can detect 18 high-risk subtypes of HPV, including 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82, 83.

Statistical analysis

Spss19.0 statistical software was used to analyze the measurement data. x ± s was used to represent the measurement data. Man Whitney rank sum test was used for the comparison between groups. Chi square test was used for the comparison between groups. The difference was statistically significant ($P < 0.05$).

Group	Primer Sequence(5′→3′)	Fragment Length/bp
DKK-1(M)	F: TTTGTAGGAAGCGTCGAAAAC	190
	R: CCGCAATATTCTCAAAACGA	
DKK-1(U)	F: TTTGTAGGAAGTGTTGAAAATG	191
	R: CCACAATATTCTCAAAACAAAA	

M: methylated, U: Unmethylated

Table 1. Primer sequence and product fragment size of DKK-1

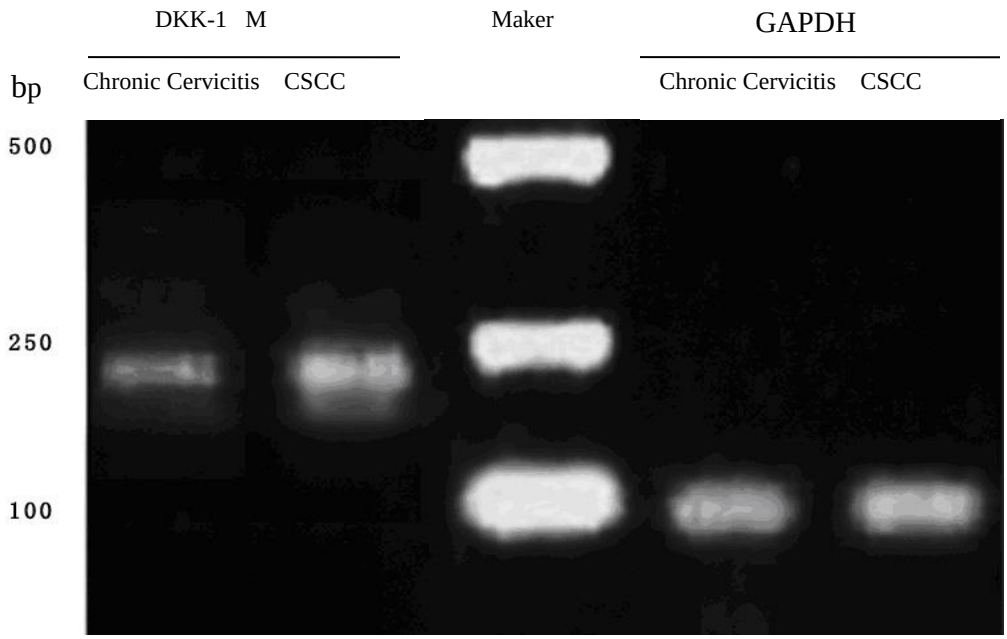

Figure 1. Electrophoretogram of methylation products of DKK-1 promoter in exfoliated cells of cervical squamous cell carcinoma

Group	Unmethylation Group	*P*	Methylation Group	*P*
Control Group	0.378±0.0158		0.277±0.0154	
Experimental Group	0.304±0.0445	0.000	0.349±0.0436	0.000

Table 2. Ratio of gray value of promoter methylation of DKK-1 gene in cervical exfoliated cells of two groups **($\bar{X}$ ±s)**

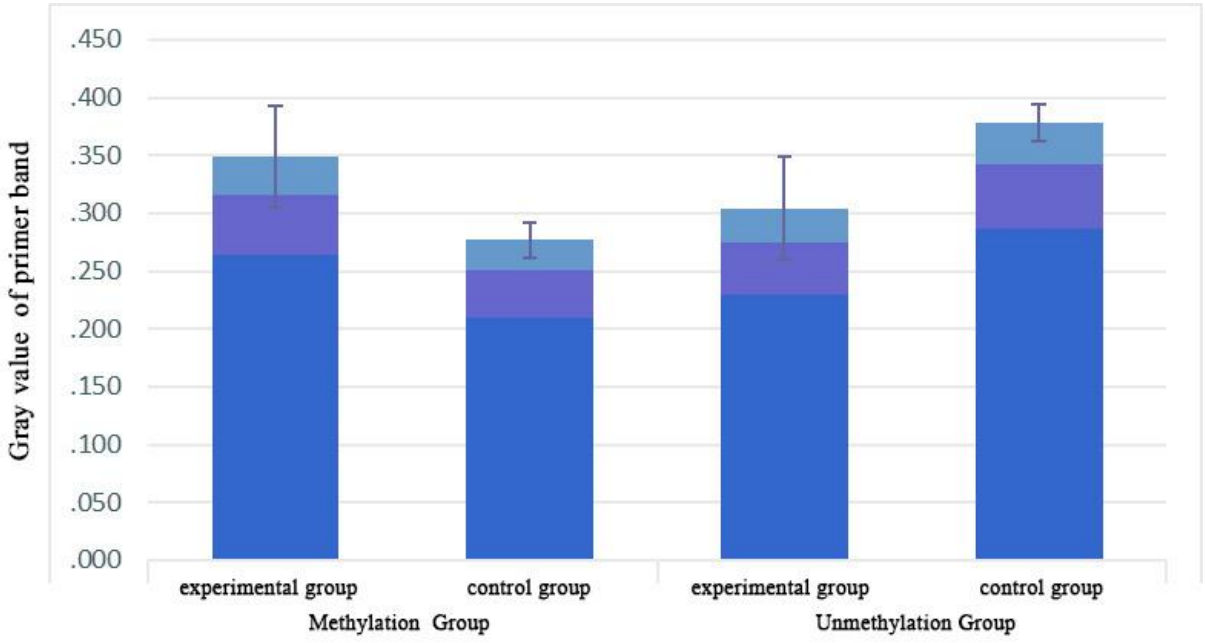

Figure 2. Comparison of gray values of methylation and unmethylation primer bands of DKK-1 gene promoter between two groups

Clinicopathological Factors	n	Methylation	Unmethylation	Methylation Rate	P
Age (years)					P＝0.6053＞0.05
≤50	15	9	6	60%	
＞50	25	20	5	80%	
High Risk HPV Infection					P＝0.0072＜0.05
Positive	34	28	6	82.4%	
Negative	6	1	5	16.7%	
Differentiation Degree					*P＝0.0126＜0.05
High Differentiation	14	5	9	35.7%	
Middle Differentiation	23	21	2	91.3%	
Low Differentiation	3	3	0	100%	
Tumor Diameter（CM）					P＝0.0383＜0.05
≤4	21	12	9	57.1%	
＞4	19	17	2	89.5%	
Lymph Node Metastasis					P＝0.0384＜0.05
Yes	16	16	0	100%	
NO	24	13	11	54.2%	
FIGO Stages					**P＝0.0264＜0.05
Phase I	15	6	9	40%	
Phase IIa	4	3	1	75%	
Phase IIb	15	14	1	93.3%	
Phase III	4	4	0	100%	
PhaseIV	2	2	0	100%	

*: High differentiation VS Middle and low differentiation **: Phase I VS Phase II and above

Table 3. Relationship between promoter methylation of DKK-1 gene and clinicopathological factors in cervical squamous cell carcinoma

HPV Type	N	Unmethylation Group	*P*	Methylation Group	*P*
16 and(or) 18 Type	26	0.288±0.0382		0.361±0.0343	
Other 16 High Risk Types	8	0.315±0.0365	0.038	0.349±0.0471	0.776

Table 4. The ratio of 16,18 types of HPV to other 16 types of high risk HPV in the experimental group **($\bar{X}$ ±s)**

Test Method	Sensitivity (%)	Specificity(%)	Accuracy(%)
High Risk HPV	85.0%	85.0%	85.0%
DKK1 Methylation	90.6%	77.1%	82.8%
DKK1 Methylation +High Risk HPV	96.7%	78.0%	85.0%

Table 5. Comparison of three detection methods in cervical exfoliated cells

Results

Comparison of methylation degree of DKK-1 gene promoter between two groups of cervical exfoliated cells

The results of MSP showed that the methylation rates of DKK-1 gene promoter in the experimental group and the control group were 72.5 % (29/40) and 12.5% (5/40), respectively. The difference between the two groups was statistically significant ($P < 0.05$). Although 5 cases in the control group were also positive, the methylation band of the target gene in the experimental group was more obvious than that in the control group, suggesting that the methylation degree of DKK-1 gene promoter in the experimental group was higher (Figure 1). The gray values of strips in the scanning images of agarose gel electrophoresis were measured and processed, and four sets of data were obtained. Data 1: The ratio of the gray value of the target gene with unmethylated primer band to the gray value of the internal reference gene band (internal reference gray value for short) in the experimental group. Data 2: The ratio of gray value of target gene with unmethylated primer band to internal reference gray value in the control group. Data 3: The ratio of the gray value of the target gene methylation primer bands of the experimental group to the internal parameter gray value. Data 4: The ratio of the gray value of the target gene methylation primer band to the internal parameter gray value in the control group.. Four groups of data were tested for normality, all $P > 0.05$, obeying normal distribution. Data 1 and 2 (unmethylated group), data 3 and data 4 (methylated group) were tested for homogeneity of variance, and the two groups had homogeneity of variance. Using Mann Whitney rank sum test to do statistical analysis on the data of the two groups, the results of the unmethylated group were: $z = -6.409$, $P = 0.000$ ($P < 0.05$). It can be considered that the gray value of the strips in the unmethylated group was statistically significant. The results of methylation group are: $z = -6.158$, $P = 0.000$ ($P < 0.05$). It can be considered that the gray value of methylation group has statistical significance (Table 2). The gray value of the control group is higher than the experimental group in the unmethylated group, and the gray value of the experimental group is higher than the control group in the methylated group (Figure 2).

The relationship between methylation of DKK-1 gene promoter and clinicopathological factors

For the statistical analysis of the sample data of the experimental group, Mann Whitney rank sum test was used. The results of the methylation group and the non methylation group were basically the same, so only the average gray value data of the methylation bands of the experimental group and the negative control group were compared. There was no significant difference in the methylation degree of DKK-1 gene promoter between the two groups ($P > 0.05$). There were significant differences in high-risk HPV infection, degree of tissue differentiation, tumor size, lymph node metastasis and FIGO stage (all $P < 0.05$) The methylation rate of DKK-1 gene promoter in cervical squamous cell carcinoma patients with high-risk HPV infection, low degree of tissue differentiation, tumor maximum diameter ＞ 4cm, lymph node metastasis and late FIGO stage was significantly higher than those without high-risk HPV infection, high degree of tissue differentiation, tumor maximum diameter ≤4cm,no lymph node metastasis and early FIGO stage. The rate of methylation in each group was calculated: (number of methylation cases/total number of cases in the group) × 100%（Table 3).

The relationship between methylation of DKK-1 gene promoter and high risk HPV infection in cervical squamous cell carcinoma

Among the 18 high-risk subtypes of HPV detected in 40 cases of cervical squamous cell carcinoma in the experimental group, 34 cases were high-risk HPV positive, while 6 cases in the control group were positive. In the experimental group, 26 cases were positive for HPV type 16 and(or) 18, and 8 cases were positive for other 16 types of high-risk HPV (non 16 or 18type). The gray value data of 16,18 and other HPV target gene bands were compared with that of internal reference bands, and the Mann Whitney rank sum test was used. The results of methylation group were: $z = -0.284$, $P = 0.776$ ($P > 0.05$); the results of non methylation group were: $z = -2.071$, $P = 0.038$ ($P < 0.05$). It can be concluded that there is no significant difference in the methylation degree of DKK-1 gene promoter between HPV-16 and HPV-18 positive patients and other 16 high-risk HPV positive patients (Table 4).

Comparison of three methods for detection of high-risk HPV in cervical exfoliated cells, methylation of DKK-1 gene promoter and their combination

The sensitivity, specificity and accuracy of detection of high-risk HPV in cervical exfoliated cells of 40 patients were all 85.0%.The sensitivity, specificity and accuracy of methylation detection of DKK-1 gene promoter were 90.6%, 77.1% and 82.8% respectively; the sensitivity, specificity and accuracy of the combined detection were 96.7%, 78.0%and 85.0% respectively (Table 5).

Discussion

Epigenetics is a subject that studies the heritability change of gene expression without the change of DNA sequence. DNA methylation is one of the main manifestations of epigenetics, which has become an important direction of precise research of tumor [7]. The main mechanism is that under the catalysis of DNA methyltransferase , the C atom at the 5 'end of CpG is covalently bound with the active methyl provided by S-adenosylmethionine (SAM), forming DNA methylation modification [3]. The methylation pattern of tumor cells shows that the extensive hypomethylation of the whole genome increases the chromosomal instability due to the activation of protooncogenes. At the same time, the hypermethylation of CpG island, which is the promoter of some genes (such as tumor suppressor genes), causes gene silencing, so as to lose the disease protective factors and promote tumorigenesis. The results show that DNA promoter methylation is an early event in the occurrence of cancer, and it is closely related to the occurrence and development of cervical cancer. It can be used as cervical cancer specific molecular markers for early diagnosis [8]. DKK-1, as an inhibitor of Wnt signaling pathway, has the characteristics of tumor suppressor gene in many human tumors. The inactivation of DKK1 gene caused by hypermethylation has been reported in gastric cancer, colorectal cancer and breast cancer, while other studies have reported that the methylation level of DKK-1 gene promoter in cervical cancer tissue is

significantly higher than that in normal tissue, and the methylation level is positively correlated with the severity of cervical cancer [6]. However, there is not any clinical study on DKK-1 gene methylation in cervical exfoliated cells of patients with cervical squamous cell carcinoma. Cervical exfoliative cytology is more noninvasive, convenient and quick than cervical histopathology, which is easy to be accepted by the majority of patients. Therefore, detection of DKK-1 gene methylation in cervical exfoliative cells may be a feasible method for noninvasive screening of cervical squamous cell carcinoma. This study showed that the methylation level of DKK-1 gene promoter in cervical exfoliated cells of patients with cervical squamous cell carcinoma was significantly higher than that in patients with chronic cervicitis, which confirmed the role of DKK-1 gene promoter methylation in the pathogenesis of cervical squamous cell carcinoma.

At present, it is believed that the condition and prognosis of patients with cervical squamous cell carcinoma are closely related to tissue differentiation, tumor size, lymph node metastasis and FIGO stage. Patients with low differentiation, large tumor, lymph node metastasis and FIGO stage later have a poor prognosis. In this experiment, the methylation rate of DKK-1 gene in the normal group (≤50 years old) and the elderly group (> 50 years old) was 60% and 80% respectively, and there was no statistical difference between the two groups, suggesting that the detection method can be used in all age samples. The methylation rate of high-risk HPV was 82.4%. The sample methylation rate of non-high risk HPV infection was 16.7%, the difference was statistically significant, suggesting that high risk HPV infection was closely related to cervical squamous cell carcinoma, which was the same as the research results of Kong TW et al. [9]. There was no significant differences in the methylation degree of DKK-1 gene promoter between the 16 and(or)18 HPV positive group and the other 16 high risk HPV positive groups. It is suggested that the methylation level of DKK-1 gene promoter is not related to the type of high-risk HPV infection. In the group of differentiation degree, the rate of hypermethylation degree in the group of high differentiation degree was significantly lower than that in the group of middle and low differentiation degree, which was statistically significant, suggesting that the inhibition of DKK-1 expression might be related to the lower degree of tissue differentiation. In the tumor diameter group, the hypermethylation rate of the larger tumor group (diameter > 4cm) was significantly higher than that of the tumor group (diameter ≤ 4cm), which was statistically significant, suggesting that the inhibition of DKK-1 expression may affect the tumor size. In addition, in the lymph node metastasis group and FIGO stage, the differences of each group were statistically significant, suggesting that the inhibition of DKK-1 expression may have a high correlation with lymph node metastasis and tumor development. Chen X et al. Showed that the inhibition of DKK-1 expression can increase the invasion and metastasis of cancer cells [10], which is consistent with our results in clinical stage and lymph node metastasis. The high methylation rate of DKK-1 gene in stage II and later stage significantly increased, suggesting that understanding the methylation and expression of DKK-1 may have a certain reference value for the progress of the disease. The above evidences indicate that the methylation of DKK-1 gene promoter in cervical exfoliated cells is closely related to the occurrence, development and prognosis of cervical squamous cell carcinoma, which may be a marker for the evaluation of its condition and prognosis, and provide clues for targeted treatment of cervical squamous cell carcinoma.

Clinical and epidemiological investigations have found that cervical cancer screening and early diagnosis can reduce the incidence rate and mortality of cervical cancer, and it is an effective means for prevention and treatment of cervical cancer. The current screening methods fail to make full use of the key molecular biological events that lead to cervical cancer, and the screening process is cumbersome, and the guidelines and standards are complex. Repeated screening for the whole population leads to high economic burden [11], It is difficult for patients to accept the invasive examination of cervical tissue, so it is very important to find the molecular biological markers with good specificity and high sensitivity in the occurrence and development of cervical cancer and apply them to the non-invasive screening of cervical cancer. Because of the prevalence of abnormal gene methylation in cervical cancer, the host gene methylation test has the advantages of higher stability, sensitivity, specificity, objectivity, consistency, and automation in the diagnosis of tumors [12]. Although the price is still high, it can improve the accuracy of screening, reduce the rate of missed diagnosis, and reduce the panic of domestic women in the hospital opportunistic screening. In this study, we found that the sensitivity, specificity and accuracy of detection of DKK-1 gene promoter methylation combined with high-risk HPV in cervical exfoliated cells were 96.7%, 78.0% and 85.0% respectively, which improved the sensitivity of diagnosis of cervical squamous cell carcinoma, and the specificity and accuracy of detection of DKK-1 gene promoter methylation were also improved compared with that of detection of DKK-1 gene promoter methylation alone, which may become non-invasive in the future cervical cancer The main direction of screening. It is necessary to further clarify the clinical application value by expanding the sample size in the future.

Conclusions

Methylation of DKK-1 gene promoter in cervical exfoliated cells of patients with CSCC is related to high-risk HPV infection and different clinicopathological factors, but the degree of methylation of DKK-1 gene is not related to the type of high-risk HPV infection. It can be used to estimate the development and prognosis of CSCC and provide clues for targeted treatment of CSCC. DKK-1 gene methylation combined with high risk HPV detection can improve the sensitivity of CSCC diagnosis, and has high specificity and accuracy, which may become a non-invasive screening method for CSCC.

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Author contributions

YH: performed the experiments, collected and analyzed the data and wrote the manuscript. RS: performed the experiments and collected the data.. JZ: designed the experiments, analyzed the data and edited the manuscript.

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Compliance with ethical standards

Ethical approval

Pessary is a noninvasive test and treatment, which is voluntarily chosen and informed about to the patients before using, so Institutional Review Board approval was exempted for the study

Conflict of interest

The authors declare that they have no conflict of interest.

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Correspondence: Jian Zheng, Department of Gynecology and Obstetrics, The Affiliated Hospital of Inner Mongolia Medical University, People’s Republic of China, E-mail: zheng_jian625@aliyun.com

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