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ORIGINAL RESEARCH

Influence of MDR1 methylation on the curative effect of interventional embolism chemotherapy for cervical cancer

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Background: Multi-drug resistance (MDR) is the main cause of tumor failure to chemotherapy. This study aims to explore the influence of MDR1 methylation on curative effect of interventional embolism chemotherapy for cervical cancer.

Methods: Sixty-seven patients with cervical cancer receiving embolism chemotherapy were selected, and 45 normal cervical tissues were included as a control. Immunohistochemistry was used to detect the level of P-glycoprotein (P-gp) in cervical cancer, and to make an analysis compared with normal tissues. The methylation status of the *MDR1* gene promoter region 16 CpG units was analyzed by using kilobase-specific cracking and matrix-assisted laser desorption ionization time of flight mass spectrometry.

Results: The results indicated that the positive expression rates of P-gp were 0% (0/45) in normal cervical tissue, and 61.19% (41/67) and 77.61% (52/67) before and after interventional embolism chemotherapy in cervical cancer tissues, respectively. There were significant differences compared with normal cervical tissues (χ^2=4.2523, 0.0392). The positive expression rate of P-gp before chemotherapy was negatively correlated with efficacy of chemotherapy (r=−0.340, P=0.005). Methylation rate of 13 CpG units in normal tissues was significantly greater than cervical tissues ($P<0.05$). In cervical cancer tissue, methylation rate of six CpG units before interventional embolism chemotherapy was higher than after chemotherapy, but that of one CpG unit was lower than after chemotherapy ($P<0.05$). The methylation rate of one CpG unit with effective chemotherapy before chemotherapy was significantly higher than ineffective chemotherapy ($P<0.05$), and the other CpG units were similar ($P>0.05$).

Conclusion: P-gp expression level coded by MDR1, methylation status of partial *MDR1* gene promoter regions CpG island, is closely related to the efficacy of interventional embolism chemotherapy for cervical cancer before the operation.

Keywords: cervical cancer, interventional embolism chemotherapy, *MDR1* gene, methylation, immunohistochemistry

Introduction

Cervical cancer is one of the most common gynecologic malignant tumors, and the morbidity is secondary to breast cancer. According to the statistics, cervical cancer takes up approximately 10% of gynecologic malignant tumor, with the yearly increasing cases of 471,000 and the yearly death cases of 275,000.[1,2] Surgery and radiotherapy are the main methods of treatment for cervical cancer. However, although surgical skills, radiotherapy equipment, and technology update in recent decades, the therapeutic effect is not enhanced and the 5-year survival rate is only 70%.[1,2] Tumor local uncontrol and reoccurrence is the main cause of treatment failure, followed by lymphatic metastasis and metastasis.

Neoadjuvant chemotherapy is defined as a certain course of chemotherapy that the patients receive before the main treatment method to shrink the tumor volume, improve the operation results, and reduce operative complications, and is especially effective in patients with adenocarcinoma insensitive to radiotherapy.[3] Cervical cancer is moderately sensitive to chemotherapy, so preoperative chemotherapy can effectively reduce tumor load, control infiltrating vessel carcinoma embolus in parametrium, reduce the radiotherapy burden, increase the operative respectability, and decrease the reoccurrence rate.[4] Neoadjuvant intra-arterial chemotherapy is the new representative treatment strategy. For this strategy, the radical trachelectomy and lymphadenectomy are done after embolism chemotherapy through the internal iliac artery and uterine artery. Also, the treatment can reserve uterine, fallopian tube, and ovaries, therefore, it is of significance to patients with early cervical cancer who want to preserve fertility. Nevertheless, lots of patients are not sensitive to interventional embolism chemotherapy, so carrying out chemotherapy for such patients will delay the overall treatment and cause unnecessary side effects and increase the financial burden.[5] Therefore, urgently seeking for indexes predicting chemo-sensitivity provides the guidance for its treatment.

Multi-drug resistance (MDR) is the main cause of tumor failure to chemotherapy. Relevant study showed that P-glycoprotein (P-gp) coded by *MDR1* gene is a marker of MDR, and the inhibition of its expression can enhance the sensitivity of certain tumor cells to chemotherapy drugs.[6,7] In this study, immunohistochemistry was used to detect the level of P-gp in cervical cancer before and after interventional embolism chemotherapy, and the methylation status of *MDR1* gene was analyzed by kilobase-specific cracking and matrix-assisted laser desorption ionization time of flight mass spectrometry, with the intention to explore the influence of MDR1 methylation on the curative effect of preoperative interventional embolism chemotherapy for cervical cancer.

Patients and methods
General data
Sixty-seven patients with cervical cancer receiving embolism chemotherapy by pathological confirmation in the Affiliated Baiyun Hospital of Guizhou Medical University from January 2013 to January 2015 were selected and all patients had not previously had radiotherapy and chemotherapy. Additionally, 45 normal cervical tissues by cervical biopsy or excision were selected as controls. This study was approved by the ethics committee of the Affiliated Baiyun Hospital of Guizhou

Medical University, Guiyang, People's Republic of China. The study adhered to the ethics guidelines of the Declaration of Helsinki. All the patients and normal individuals gave their informed, written consent and approved the present study.

Level of P-gp in cervical cancer tissues detected by immunohistochemistry
Cervical cancer and normal cervical tissues before and after interventional treatment were embedded into paraffin, cut into 5 μm slices, and conventional xylene dewaxing and gradient alcohol hydration was performed. Then the tissues were endogenously peroxidase sealed by 3% hydrogen peroxide solution, distilled water to rinse three times, and citrate buffer boiling repair antigen. The first antibody (mouse anti-P-gp monoclonal antibody purchased from Abcam PLC [Cambridge, UK]) was added and put in a humidified box at 4°C for staying overnight, washed with Tris-buffered saline for three times, diaminobenzidine developing (DAB Chromogenic Reagent Kit by ZSGB-BIO, Beijing, People's Republic of China), hematoxylin staining for 1 minute, dehydration and transparency, and finally sealed by neutral balsam. The known positive biopsy was regarded as a positive control and phosphate-buffered saline instead of the primary antibody was considered as a control.

Detection of the methylation status of *MDR1* gene
The extraction and identification of DNA
Cervical cancer and normal cervical tissues during 2–3 weeks before and after interventional treatment were taken for DNA extraction by QIAamp DNA Mini Kit (Qiagen NV, Venlo, the Netherlands), strictly according to specification. The house-keeping gene β-globin (primer sequences: forward: 5′-CAACTTCATCCACGTTCACC-3′; reverse: 5′-GAAGAGCCAAGGACAGGTAC-3′) was the internal control for PCR amplification. The PCR-amplified product was confirmed by sequencing as the positive cervical samples of β-globin DNA (positive control) and sterilized double-distilled water (ddH$_2$O) was designed as blank control. PCR product was identified by 2% agarose gel electrophoresis. If the product is obviously seen, the quality of DNA was passed. The DNA concentration and purity was detected by the ultraviolet photometer.

The methylation process: PCR and in vitro transcription
DNA was processed by hydrosulfite according to kit introduction and then performed with PCR. The EZ DNA

methylation kit was purchased from the Zymo Research Corporation (Irvine, CA, USA). The reaction system was 3 μL, including 0.9 μL ddH$_2$O, 0.5 μL 10× PCR buffer (Mg^{2+}), 0.4 μL MgCl$_2$, 0.1 μL 25 mM dNTP, 1 μL primer mix, and 0.1 μL 5 U/μL PCR enzyme. *MDR1* gene sequences of primers: forward: 5′-TTGGAGGTGAGATTAATTT-3′b; reverse: 5′-AAAAAAACCCCTACAATACCCATC-3′c, b and c are primers, prefix of primers, that is, each forward primer marks ten base sequence (5′-AGGAAGAGAG-3′) and each reserve primer marks a T7 promoter (5′-CAGTAA TACGACTCACTATAGGGAGAAGGCT-3′). The reaction condition was 94°C for 4 minutes, 94°C for 20 seconds, 56°C for 30 seconds, 72°C for 1 minute, 45 cycles in total, and last 72°C for 3 minutes. After the reaction, 5 μL PCR product was taken and added with 2 μL shrimp alkaline phosphatase mixture (including 0.3 μL shrimp alkaline phosphatase and 1.7 μL ddH$_2$O), centrifuged and incubated at 37°C for 20 minutes, then at 85°C for 5 minutes for eliminating the free nucleotide in the reaction system. The product (2 μL) was put in microwell plate, added with 5 μL transcription/RNase A mixture (including 3.15 μL RNase-free ddH$_2$O, 0.89 μL 5× Tris (T) and polymerase buffer, 0.24 μL T cleavage mix, 0.22 μL 100 mM DL-Dithiothreitol (DTT), 0.44 μL T7 RNA and DNA polymerase, and 0.06 μL RNase A), sealed, and centrifuged. The product was finally incubated at 37°C for 3 hours, and preserved at 4°C.

Detection of methylation rate

The methylation status of the *MDR1* gene promoter region of 16 CpG units was analyzed by kilobase-specific cracking and matrix-assisted laser desorption ionization time of flight mass spectrometry. Nanodispenser was used to take 22 nL lytic reaction liquid to add to matrix chip, and MassARRAY (Sequenom, San Diego, CA, USA) was used to collect mass spectrum. The methylation rate of mass spectrum was analyzed by Epityper 1.0 software.

Evaluation criteria

Evaluation for chemotherapy curative effect

The three-dimensional radial line was detected by transvaginal ultrasonography during 2–3 weeks before and after chemotherapy for calculating the volume of tumor, mainly marked by incrassation in parametrium, loss of normal cervical morphology, disappeared sacrum cardinal ligament. Parametric infiltration was calculated according to the unilateral side. In accordance with International Union Against Cancer, criteria of curative effect include complete remission, partial remission, stable disease, and progressive disease. Complete

remission and partial remission refer to effective, and stable disease and progressive disease refer to ineffective.

Evaluation for P-gp expression level

P-gp positive particles showed brownness in the membrane and cytoplasm. Four visual fields were selected under the high-power lens for counting 200 cells. The number of positive cells <10% indicated with "–", 10%–25% indicated with "+", >25%–75% indicated with "++", and >75% indicated with "+++". + to +++ refers to the positive expression.

Statistical data analysis

SPSS 17.0 software was used for data analysis. The enumeration data were expressed as a percentage (%), the rate of groups was analyzed by χ^2 test, and the correlation was by Spearman's rank correlation analysis. The methylation levels between cervical cancer tissue and normal cervical tissue, before and after treatment for cervical cancer tissues were analyzed by the Wilcoxon rank sum test. A value of $P < 0.05$ was considered statistically significant.

Results

Demographics of patients

The patients were aged 27–68 years, with the median age of 53 years. According to the cervical cancer clinical staging of International Federation of Gynecology and Obstetrics, the patients were divided into Ib2, IIa, IIb, IIIa, and IIIb stage, which were listed in Table 1. All the patients, 48 patients with squamous carcinoma and 19 patients with adenocarcinoma, were also divided into poorly differentiated, moderately differentiated, and highly differentiated degree (Table 1).

Table 1 Demographics of the patients involved in this study

Demographics	Values
Age (years), mean (range)	53 (27–68)
FIGO stage, n	
Ib2	21
IIa	16
IIb	13
IIIa	10
IIIb	7
Cancer type, n	
Squamous carcinoma	48
Adenocarcinoma	19
Differentiated degree, n	
Poor	4
Moderate	24
High	39

Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.
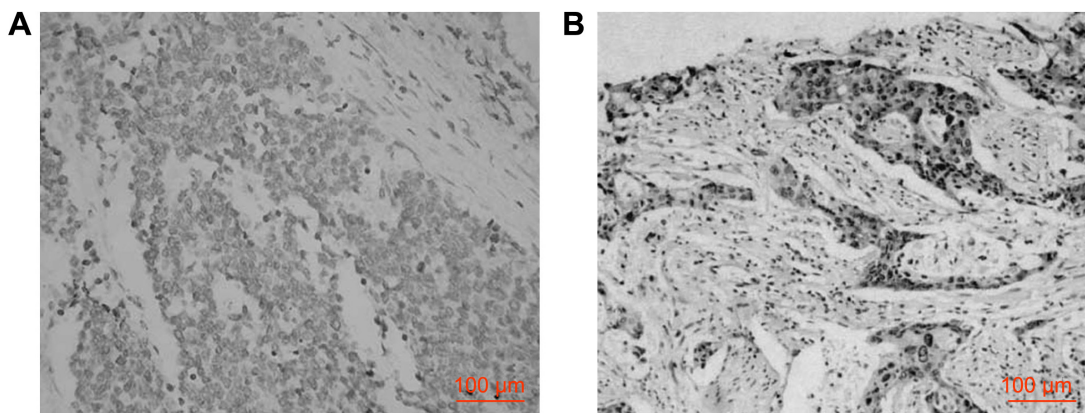
Figure 1 Expression of P-glycoprotein in normal cervical tissues and cervical cancer tissues.
Notes: (**A**) Normal cervical tissues; (**B**) cervical cancer tissue.

The P-gp expression level in normal tissues and cervical cancer tissues before and after interventional embolism chemotherapy

The positive expression rate of P-gp was 0% (0/45) in the normal cervical tissues, 61.19% (41/67) and 77.61% (52/67) in cervical cancer tissues before and after interventional embolism chemotherapy. The positive expression rate of P-gp after interventional embolism chemotherapy was higher than after interventional embolism chemotherapy (χ^2=4.2523, P=0.0392). The expression intensity of P-gp after chemotherapy increased in cervical cancer tissues compared with before chemotherapy before (χ^2=17.7301, P=0.0005) (Figure 1 and Table 2).

Influence of P-gp expression on chemotherapeutic effect before chemotherapy

The positive expression rate of P-gp before chemotherapy was negatively correlated with chemotherapeutic effect (r=−0.340, P=0.005). The positive expression rate of P-gp before chemotherapy in patients with effective chemotherapy was lower than in patients with invalid chemotherapy (χ^2=7.7274, P=0.0054) (Table 3).

The identification and purification of DNA

After β-globin PCR product was performed with 2% agarose gel electrophoresis, the target fragments appeared at 240 bp, as shown in Figure 2. Ultraviolet absorbency degree OD260/OD280 was 1.8–2.0, with DNA concentration >50 ng/µL, applicable for PCR amplification.

Methylation status of MDR1 in normal tissues and cervical cancer tissues before and after chemotherapy

Mass spectrum accurately positioned methylation status of each CpG unit of gene segment. Each sample amplicon in the present study included 16 CpG units, and each CpG unit included single or multiple CpG loci, as shown in Table 4. The methylation rate of 13 CpG units (Unit 2–Unit 11 and Unit 14–Unit 16) in normal cervical tissue was higher than cervical cancer tissues (P<0.05). The methylation rate of six CpG units (Unit 4, Unit 5, Unit 8–Unit 10, Unit 16) was higher in cervical cancer tissue before chemotherapy than after chemotherapy, but the methylation rate of Unit 14 was lower than after chemotherapy (P<0.05) (Figure 3).

Table 2 Comparison of P-gp expression level in normal tissues and cervical cancer tissues before and after interventional embolism chemotherapy

	Tissue samples, n	P-gp expression level, n (%)				χ^2	*P*-value
		(−)	(+)	(++)	(+++)		
Before chemotherapy	67	26 (38.81)	24 (35.82)	10 (14.93)	7 (10.45)	17.7301	0.0005
After chemotherapy	67	15 (22.39)	11 (16.42)	25 (37.31)	16 (23.88)	NA	NA

Notes: "−" refers to <10% positive cells; "+" refers to 10%–25% positive cells; "++" refers to >25%–75% positive cells; and "+++" refers to >75% positive cells.
Abbreviations: NA, not applicable; P-gp, P-glycoprotein.

Table 3 Correlation between the expression of P-gp before chemotherapy and chemotherapeutic effect

Efficacy	Tissue samples, n	P-gp expression level, n (%)		χ^2	*P*-value
		Negative	Positive		
Effective	52	28 (53.85)	24 (46.15)	7.7274	0.0054
Ineffective	15	2 (13.33)	13 (86.67)	NA	NA

Abbreviations: NA, not applicable; P-gp, P-glycoprotein.

Influence of MDR1 methylation status on chemosensitivity

The methylation rate of CpG_2, 3, and 4 loci before chemotherapy in patients with effective chemotherapy was higher than patients with ineffective chemotherapy, and there was no difference in the methylation rate among the other CpG loci (*P*>0.05) (Figure 4).

Discussion

The *MDR* gene family includes *MDR1* and *MDR3*, but only *MDR1* gene can produce MDR phenomena. *MDR1* gene is located in 7q21.1–q21.3 loci on chromosome 7, including 29 exons and 28 introns and coding drug carrier P-gp.[8] P-gp, one of the members in the superfamily of ATP-binding cassette transporters, has energy-dependent expanding membrane efflux pumping function, pumps a variety of antitumor drugs out of cells, makes intracellular
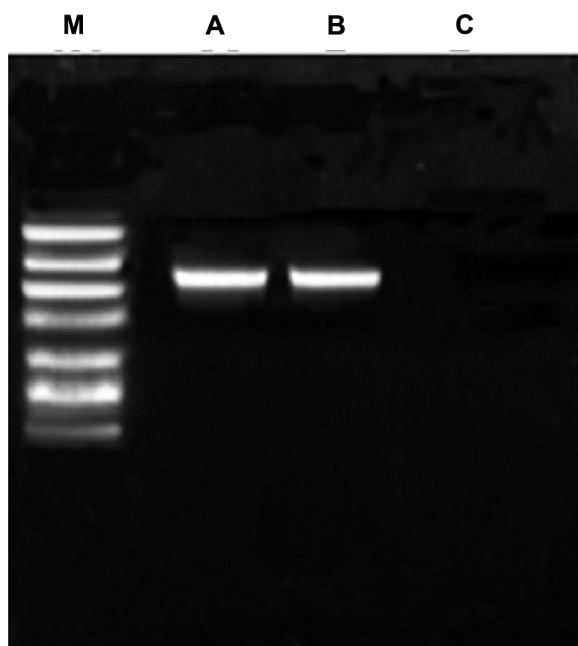

Figure 2 β-Globin PCR product electrophoretogram.
Notes: M, DNA marker; A, sample; B, positive control; C, blank control.
Abbreviation: PCR, polymerase chain reaction.

Table 4 CpG units and the corresponding CpG loci in amplicon

CpG units	CpG loci
Unit 1	CpG_2,3,4
Unit 2	CpG_5
Unit 3	CpG_6
Unit 4	CpG_7
Unit 5	CpG_8
Unit 6	CpG_9,10
Unit 7	CpG_11
Unit 8	CpG_12,13
Unit 9	CpG_18
Unit 10	CpG_19,20
Unit 11	CpG_22
Unit 12	CpG_23
Unit 13	CpG_24
Unit 14	CpG_25,26,27
Unit 15	CpG_28
Unit 16	CpG_30

chemotherapeutics fall short of effective dose, thus producing drug resistance.[9–11] MDR produced by the drug efflux function of P-gp-mediated tumor is close to the level of P-gp expression. P-gp is lowly expressed in tumor cells with drug susceptibility and highly expressed in drug-resistant tumor cells.[12] Scheiner et al[13] detected the P-gp expression level in 109 patients with acute myeloid leukemia, and the results showed that the expression of P-gp was negatively correlated with the treatment remission rate, hence, it was the poor prognostic factor. Zhu et al[14] used immunohistochemical method to detect the expression of P-gp in 82 patients with breast cancer and analyzed the relationship between the clinicopathological features and the prognosis of the patients, the results showed that the P-gp expression level was closely associated with the prognosis of patients with breast cancer, the higher the expression level, the lower the survival rate. In the present research, P-gp was not expressed in normal cervical tissues and highly expressed in cervical cancer tissues, and the positive expression rate of P-gp before chemotherapy in patients with effective chemotherapy was lower than in patients with invalid chemotherapy, which was consistent with the results of the previous studies. Additionally, the present study also revealed that the P-gp expression level significantly increased after interventional embolism chemotherapy, indicating chemotherapy could induce the expression of P-gp in cervical cancer tissues and P-gp might be close to secondary resistance.

DNA methylation is a potential biomarker of tumor. Generally, DNA methylation can inhibit the gene expression and the hypo-methylation of the whole genome is one of the most important characteristics of tumor cells.[15] The
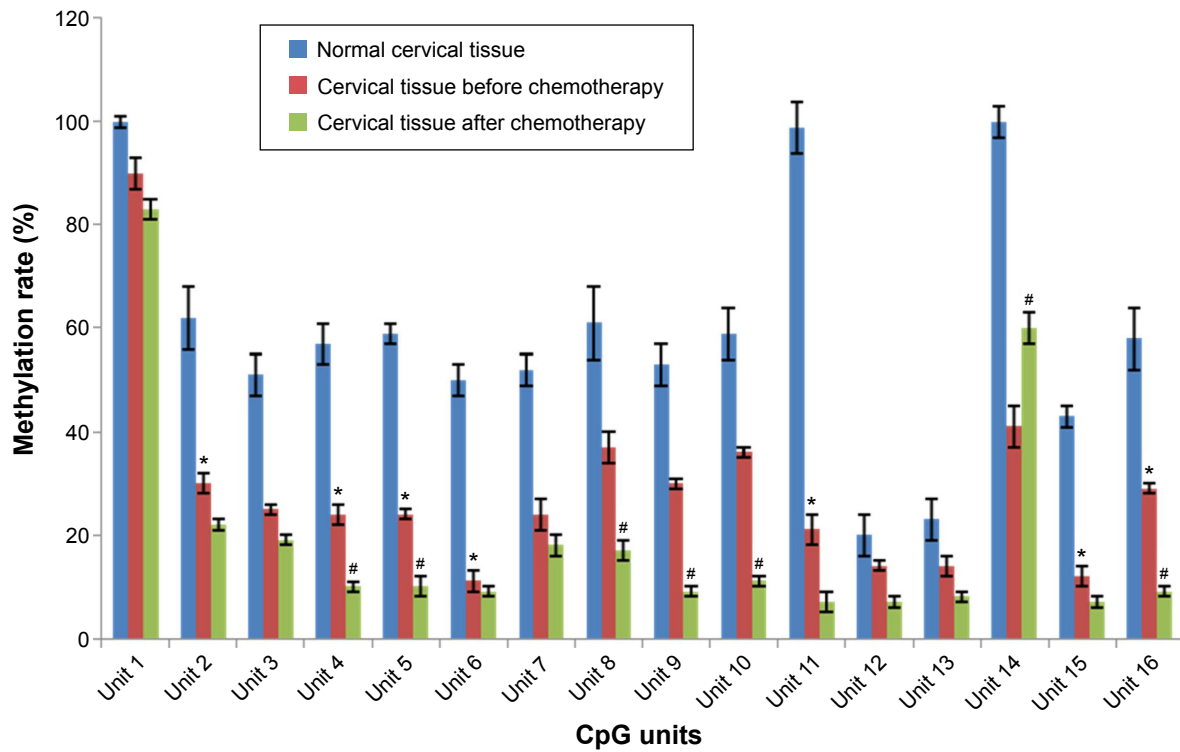
Figure 3 Mean methylation rate of *MDR1* gene CpG units in normal cervical tissues and cervical cancer tissues before and after chemotherapy.
Notes: *Statistical significance compared with normal cervical tissues; #statistical significance compared with cervical cancer tissues before chemotherapy.

MDR1 hypo-methylation leads to the highest expression of P-gp of tumor cells, consequently reducing the sensibility of tumor cells to chemotherapeutics.[16] Bebek et al[17] held that the methylation level of the MDR1 promoter region in head and neck squamous cell carcinoma, which was different

from normal mucosal tissue, was closely related to lymphatic metastasis in tumor region.

In the present study, the methylation rate of six CpG units was higher in cervical cancer tissue before chemotherapy than after chemotherapy, and the methylation rate
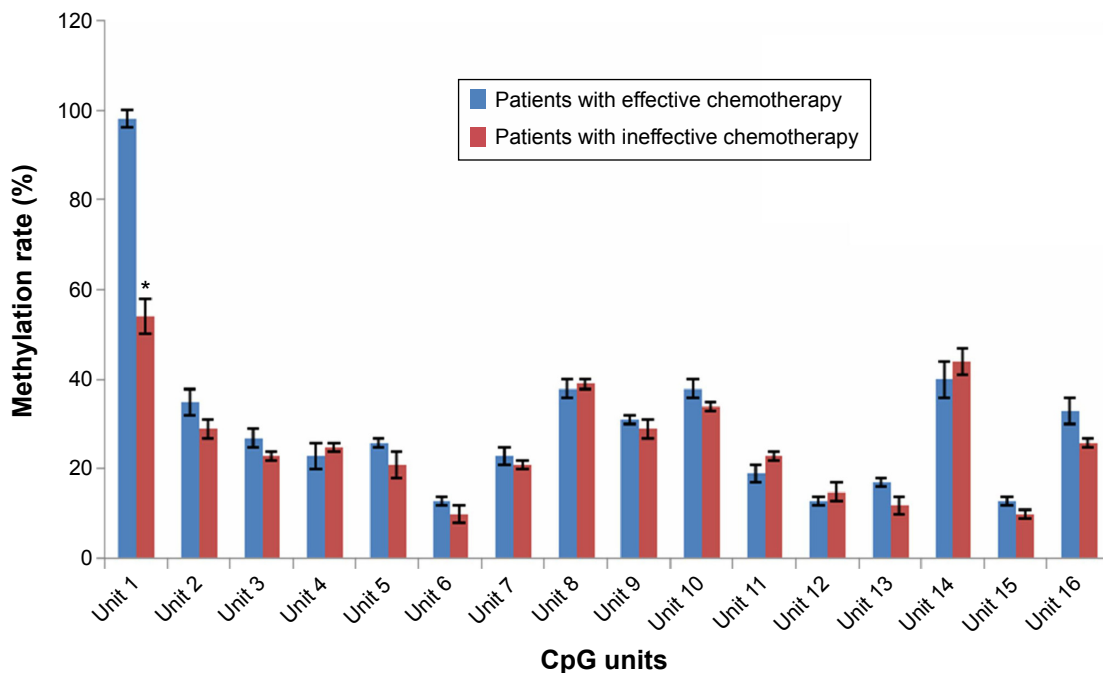
Figure 4 Mean methylation rate of *MDR1* gene CpG units in patients with different therapeutic effect of chemotherapy.
Note: *Statistical significance compared with patients with effective chemotherapy.

of CpG_2, 3, and 4 loci before chemotherapy in patients with effective chemotherapy was higher than in patients with ineffective chemotherapy, indicating that chemotherapy could change the methylation status of *MDR1* gene and the chemotherapeutic effect had a certain correlation with the methylation status of *MDR1* gene. In addition, the mean methylation rate of 13 CpG units in normal cervical tissue was higher than cervical cancer tissues ($P<0.05$), revealing the quantitative methylation of MDR1 CpG units might be a molecular marker of cervical cancer and further providing guidance for identifying cervical cancer.

Although we obtained some of the interesting results, there were also some limitations. First, whether the methylation status of those CpG loci of *MDR1* gene is able to identify the malignant degree also needs to be further studied. Second, whether the CpG loci of MDR1 could be used for the clinical diagnosis of tumors still needs to be further explored.

Conclusion

This study proves that the methylation status of the partial *MDR1* gene has certain correlation with the chemotherapeutic efficacy of interventional embolism chemotherapy for cervical cancer before operation, at the same time, it can predict the drug sensitivity of tumor to some extent, and provide the theoretical basis for individualized treatment for cervical cancer.

Disclosure

The authors report no conflicts of interest in this work.

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